UNIVERSITY OF ALBERTA

INDUCTION AND REGULATION OF DELAYED TYPE HYPERSENSITIVITY BY CD4 AND CD8 T SUBSETS

BY

LI LI ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

IN

MEDICAL SCIENCES-IMMUNOLOGY

EDMONTON, ALBERTA
FALL, 1997

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-23017-1

Canada

UNIVERSITY OF ALBERTA

LIBRARY RELEASE FORM

NAME OF AUTHOR: **LI LI**

TITLE OF THESIS **INDUCTION AND REGULATION OF DELAYED TYPE HYPERSENSITIVITY BY CD4 AND CD8 T SUBSETS**

DEGREE: **DOCTOR OF PHILOSOPHY**

YEAR THIS DEGREE GRANTED: **1997**

Permission is hereby granted to the university of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis , and except as hereinbefore provided neither the thesis nor any substantial portion thereof may be printed or other wise reproduced in any material from what ever without the author's prior written permission.

Li Li

7/15 1997

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **INDUCTION AND REGULATION OF DELAYED TYPE HYPERSENSITIVITY BY CD4 AND CD8 T SUBSETS** submitted by **LI LI** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in MEDICAL SCIENCES-IMMUNOLOGY

Dr. Timothy R. Mosmann (Supervisor)

Dean Befus

Dr. Dean Befus

Dr. John F. Elliott

Dr. David Rayner

Dr. Mike Belosevic

Dr. Philip W. Askenase (External Reviewer)

7/12

1997

TO THE MEMORY OF MY GRAND FATHER MR. HONG-YUAN LI

ABSTRACT

DTH is an in vivo manifestation of cell-mediated immune response. T cells and their cytokines are important participants and regulators of DTH response. In this study, I investigated the activities of T cell cytokine subsets in DTH induction, and characterized the regulatory effects of their cytokines and other molecules on DTH.

IL-10 is a Th2 cytokine that inhibits Th1, macrophage and neutrophil cytokine synthesis. The effects of IL-10 on DTH were tested. Mouse rIL-10 was purified to > 90% homogeneity. The IL-10 *in vivo* half life was 2 to 5 hr. Systemically administered IL-10 inhibited footpad swelling induced by Th1 clones. The inhibition was accompanied by a similar suppression of tissue edema. IL-10 inhibited the levels of IL-2, IL-6, IL-10, IFNγ and TNFα/LT in DTH footpads. IL-10 also inhibited footpad swelling and TNF/LT induced in SRBC DTH.

The ability of the CD8 Tc1 and Tc2 cell to induce DTH was tested. Allo-reactive Tc1 and Tc2 cells induced comparable levels of antigen-specific footpad swelling with similar time courses. They induced similar levels of footpad edema and infiltration of macrophages and neutrophils. Furthermore, Tc2 cells recruited more eosinophil infiltration. Tc1 and Tc2 cells retained their distinct in vitro cytokine profiles in the injected footpads and induced similar inflammatory cytokines. However IFNγ and IL-4 were not the critical mediators of the Tc1 or Tc2 DTH. Tc1 and Tc2 cells also induced tissue swelling when injected into footpads of syngeneic mice together with allo-APCs. Furthermore, Tc1 and Tc2 cells were able to migrate to local inflammatory sites. Together these results suggested that both Tc1 and Tc2 cells are able to mediate DTH during certain immune responses.

The contribution of perforin-mediated cytotoxic activity to DTH was studied. Perforin-deficient Tc1 or Tc2 cells were able to induce DTH, although at a lower magnitude. Perforin-deficient Th1 and Th2 cells induced similar footpad swelling as their control counterparts. Perforin-deficient cells produced similar levels of cytokines as did their control counterparts. Th1 cells produced similar levels of cytokines as did Tc1 cells in the injected footpads, Th2 cells produced only one tenth of cytokine levels in the Tc2 footpads. Therefore, the poor activation of Th2 cells in vivo may account for their lower ability to induce DTH.

ACKNOWLEDGMENTS

I would like to take this opportunity to express my deepest gratitude to the following people who have provided me help and support during the time of my Ph.D. training.

First, I would like to thank my supervisor Dr. Timothy R. Mosmann who led me into the wonderful world of immunological research. His dedicated and excellent supervision, patience and encouragement made this thesis possible. His serious attitude toward science and unique philosophical thinking about science have made a great influence on me during the past five years, and will continue to benefit me on my future career. Thank you Tim!

I would like to thank all the members of my supervisory and examining committee, late Dr. Tom Wegmann, Drs. Dean Befus, John Elliott, David Rayner and Mike Belosevic for the time and effort they have spent in discussing my projects, providing suggestions, offering technical helps, and giving critical readings of my thesis. A special thank to my external reviewer Dr. Phillip W. Askenase for taking time in his busy schedule to work on my thesis and defense.

I would like to thank Rita Macott for her excellent technical assistance, Yew-Hon Lai, Drs. Jean-Marine Hesenlan, Subash Sad, Peter Akai for their critical discussions on my projects and technical helps on my experiments .

I would like to thank Wilma Suarez-pinzon for helping me on immunohistochemical staining. Dr. John Elliott for providing me the IL-10 transfected cell line, Drs. David Kagi and Hans Hengartner for sending me the perforin deficient mice and Dorothy Rutkowski for FACS sorting the T cells used in my experiments.

I am indebted to my grandparents Mr. Hong-Yuan Li and Ms. Feng-Xiang Qu, my parents Mr. Fu-Heng Li and Prof. Wen-Rong Zhao for their unlimited love, unconditional support and everlasting encouragement. A special thank to my husband Dr. Lei Qiao for his love, patience, support, and particularly, for his encouragement during every difficult time.

Through out most of my graduate studies, I have been supported by Faculty of Medicine 75th Anniversary Graduate Studentship and a full-time studentship from the Alberta Heritage Foundation of Medical Research for which I am extremely grateful.

TABLE OF CONTENTS

CHAPTER **PAGE**

I. Introduction

A. Prologue....1

B. T cytokine subtypes and their roles in immune regulation....3

-Cytokines....3

-General introduction of Th1 and Th2 cells....3

-Functions of Th1 and Th2 cytokines....5

-in vivo functions of Th1 and Th2 cells during immune responses....9

-Counter-regulations between Th1 and Th2 cells during immune regulation....12

-CD8 T subsets and their functions....17

C. Delayed type hypersensitivity (DTH)....20

-Concept, physiological significance and detection of DTH....20

-Classification of DTH responses....22

-The process and the molecular and cellular participants of DTH....25

-Adoptively transferred DTH....36

II. Rationale and objectives

A. Rationale....39

B. Objectives....41

III. Materials and Methods

A. Materials and Methods (General)

-Animals....42

-T cell clones and cell lines....42

-Reagents...43
-Cytokine ELISA...44
-Cytokine bioassays..44
-Measurement of footpad swelling..44

B. Materials and Methods Specific for Chapter IV

-Recombinant IL-10 purification..45
-IL-10 western blot..46
-Measurement of the in vivo half live of IL-10.........................46
-Th1 DTH assay...47
-SRBC DTH assay..48
-Footpad histology..48
-Footpad cytokine extraction..48
-Measurement of vascular permeability....................................48

C. Materials and Methods Specific for Chapter V

-CD8 T cell purification...49
-Allo-reactive Tc1 and Tc2 cell generation................................49
-Tc1 and Tc2 cloning...50
-CD4 T cell purification..50
-Allo-reactive Th1 and Th2 cell generation................................51
-The CD8 and CD4 DTH assay..51
-Measurement of vascular permeability.......................................52
-Footpad cell extraction..52
-Immunohistochemical staining..53
-Systemically transferred DTH assay..54
-Tc1 and Tc2 migration assay..54
-Footpad cytokine extraction...54
-Footpad cytokine detection by ELISA...55

-Anti-cytokine antibodiy effects on DTH induced by Tc1 or Tc2 cells..................55

-Generation of cytokine-deficient Tc1 cells..55

D. Materials and Methods Specific for Chapter VI

-Generation of Th1, Th2, Tc1 and Tc2 cells from perforin-deficient mice............56

-Cytotoxicity assay...56

IV. IL-10 inhibits Th1 clone- and SRBC-induced footpad DTH

A. Introduction and rationale..58

B. Results

-JE 9-2 cells produces high levels of mouse rIL-10 which is biologically active and glycosylated..61

-Purification of mouse recombinant IL-10 produced by JE 9-2 cells...................62

-Measurement of the in vivo half life of JE 9-2 IL-10...64

-IL-10 inhibits M264-15 Th1 clone induced footpad swelling.................................65

-IL-10 inhibits DTH induced by other Th1 clones..68

-IL-10 inhibits vascular permeability during the Th1 DTH response....................68

-IL-10 does not alter the cellular infiltration in 24 hr DTH footpads...................69

-IL-10 reduces the level of Th1 and inflammatory cytokines in the DTH footpads..70

-IL-10 inhibits SRBC induced footpad DTH...71

C. Summary of results...73

D. Discussion..74

E. Tables and figures...81

V. CD8 Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo, but induce similar footpad DTH

A. Introduction and rationale...108

B. Results

-Both Tc1 and Tc2 cells induce adoptively transferred footpad DTH.................111

-DTH induced by Tc1 and Tc2 cells is antigen-specific, and does not require host T cells..113

-Tc1 and Tc2 cells induce similar edema during the footpad reaction................114

-Cellular infiltration in the footpads injected with Tc1 or Tc2 cells....................114

-Cytokine synthesis in vivo during DTH induced by Tc1 or Tc2 cells.................117

-Anti-cytokine antibody treatment of DTH induced by Tc1 and Tc2 cells...........119

-Lack of correlation between Tc1 cytokine synthesis and DTH induction..........119

-Footpad swelling is induced in syngeneic mice by injecting Tc1 or Tc2 cells together with APCs..120

-Systemically transferred Tc1 or Tc2 cells induce only marginal footpad swelling...121

-Both Tc1 and Tc2 cells preferentially migrate to inflamed sites........................121

C. Summary of the results..123

D. Discussion...124

E. Tables and figures..128

VI. Effects of perforin-mediated cytotoxicity on DTH induced by CD8 or CD4 T subsets

A. Introduction and rationale..166

B. Results

-Perforin-mediated cytotoxicity enhances but is not critical for DTH induced by Tc1 and Tc2 cells...170

-Perforin-mediated cytotoxicity dose not affect the footpad DTH induction by Th1 or Th2 cells...171

C. Summary of the results..174

D. Discussion....175
E. Tables and figures....178

VII. General discussion and future projects

A. General discussions....187
B. Future Directions....197
VIII. Bibliography....202

LIST OF TABLES

Table	Description	Page
4.1	Purification of mouse rIL-10	106
4.2	IL-10 inhibits cytokine levels in the DTH footpads	107
5.1A	Footpad swelling induced by Tc1 clones	164
5.1B	Footpad swelling induced by Tc2 clones	165

LIST OF FIGURES

Figure	Description	Page
3.1	Quality control of footpad measurement	57
4.1.	JE 9-2 cells produce high levels of IL-10	81
4.2.	JE 9-2 IL-10 is biologically active	82
4.3.	JE 9-2 IL-10 monomers have similar molecular weights to those of the COS cell expressed mouse rIL-10	83
4.4.	IL-10 purification by Mono Q and 3GA blue dye columns	84
4.5.	The purity of IL-10 after each step of purification	85
4.6.	Kinetics of in vivo loss of IL-10	86
4.7.	The dose effect of single i.p. injected IL-10 on DTH induced by M264-15 cells	88
4.8.	The dose effect of triple i.p. injected IL-10 on DTH induced by M264-15 cells	89
4.9.	The frequency effect of IL-10 administration on DTH induced by M264-15 cells	90
4.10.	The time effect of IL-10 inhibition on DTH induced by M264-15 cells	91
4.11.	IL-10 inhibits DTH induced by different numbers of M264-15 cells	92
4.12.	IL-10 administered by different systemic routes inhibits DTH induced by M264-15 cells	93
4.13.	IL-10 inhibits DTH induced by other Th1 clones	94

4.14. Time-course analysis of the IL-10 suppression of DTH induced by Th1 clones....95
4.15. IL-10 inhibits tissue edema in DTH induced by M264-15 cells....97
4.16. IL-10 does not alter cellular infiltration in DTH induced by M264-15 cells....98
4.17. IL-10 inhibits cytokine levels in DTH footpads....99
4.18. The time course of cytokine levels in DTH footpads and the inhibitory effect of IL-10 on these cytokines....100
4.19. IL-10 does not show synergistic effect with anti-IFNγ in DTH inhibition....101
4.20. IL-10 inhibits the effector stage of DTH induced by priming and challenging with SRBCs....102
4.21. Smaller amounts of IL-10 administered by triple i.p. injections does not significantly inhibit DTH induced by SRBCs....103
4.22. IL-10 does not alter cellular infiltration in SRBC-induced DTH....104
4.23. IL-10 inhibits levels of TNF/LT in SRBC DTH footpads....105

5.1. Allo-reactive Th1 cells are more active than Th2 cells to induce footpad DTH....128
5.2. Cytokine profiles and phenotypes of Tc1 and Tc2 cells....129
5.3. Both Tc1 and Tc2 cells induce cell dose-dependent footpad swelling....130
5.4. Tc1 and Tc2 clones induce footpad swelling....131
5.5. Footpad swelling induced by resting or activated Tc1, Tc2, Th1 and Th2 cells....132
5.6. Footpad swelling induced by Tc1 and Tc2 cells is antigen specific....133

5.7. Significant footpad swelling is induced in H2$^{d/b}$ as well as in H2^{d} mice by either Tc1 or Tc2 cells (H2^{b}-anti-H2^{d})........134

5.8. Endogenous T cells are not required for the footpad reaction induced by either Tc1 or Tc2 cells........135

5.9. Tc1 and Tc2 cells induce similar footpad edema........136

5.10. Similar granulocyte infiltration is detected in the Tc1- and Tc2-injected footpads........137

5.11. Similar macrophage infiltration is detected in the Tc1- and Tc2-injected footpads........139

5.12. Anti-Gr-1 staining of cells extracted from Tc1- or Tc2-injected footpads........141

5.13. Similar extent of neutrophil infiltration is detected from Tc1- and Tc2-injected footpads........143

5.14. Eosin Y and Methylene Blue staining of cells extracted from Tc1- or Tc2-injected footpads........145

5.15. Anti-Mac-3 staining of cells extracted from Tc1- or Tc2-injected footpads........146

5.16. Similar extent of macrophage infiltration is detected from Tc1- or Tc2-injected footpads........148

5.17. More infiltrated eosinophils are detected in Tc2-injected footpads........149

5.18. Time course of Tc1 and Tc2 cell induced eosinophil infiltration........150

5.19. Tc1 and Tc2 cells retained their in vitro cytokine profiles in the injected footpads........151

5.20. Similar levels of inflammatory cytokines are detected in Tc1- and Tc2-injected footpads........153

5.21. Positive correlation between the extent of footpad swelling and the levels of IL-6 or TNF/LT in the corresponding footpads........154

5.22. Similar Tc1 or Tc2 cytokine profiles are detected in DTH footpads of F1 (H2$^{d/b}$) mice as in BALB/c (H2^{d}) mice....................................155

5.23. Inflammatory cytokines are detected in DTH footpads of F1 (H2$^{d/b}$) mice as well as BALB/c (H2^{d}) mice..156

5.24. Antibody to IL-4 or IFNγ does not inhibit the footpad swelling induced by either Tc1 or Tc2 cells..157

5.25. Tc1 and the cytokine-deficient Tc1 (Tc1^{-}) cells induce similar footpad DTH..158

5.26. Tc1^{-} cells produce less IFNγ in vivo but induce similar footpad DTH as normal Tc1 cells...159

5.27. Lack of correlation between the in vivo IFNγ levels and the magnitudes of the footpad swelling induced by Tc1 cells..................................160

5.28. Tc1 and Tc2 cells together with target cells induce footpad swelling in syngeneic mice..161

5.29. Tc1 and Tc2 cells induce marginal footpad swelling when transferred systemically into syngeneic mice..162

5.30. Both Tc1 and Tc2 cells preferentially migrate to inflamed sites.............163

6.1. Perforin-deficient Tc1 and Tc2 cells produce similar patterns of cytokines as their normal counterpart, but do not mediate rapid killing of target cells...178

6.2. Perforin-deficient Tc1 and Tc2 cells induced similar or decreased footpad welling compared to their normal counterparts............................179

6.3. Perforin-deficient Tc1 and Tc2 cells induce cell dose-dependent footpad swelling..180

6.4. Similar cytokines are detected in the DTH footpads injected with perforin-deficient Tc cells as in those injected with their normal counterparts....181

6.5. Perforin-deficient Th1 and Th2 cells produce similar cytokines as their normal counterparts, and Th1 but not Th2 cells express moderate perforin-mediated cytotoxicity....182

6.6. Perforin does not affect the ability of either Th1 or Th2 cells to induce DTH....183

6.7. Both normal and perforin-deficient Th1, but not Th2 cells induce cell dose dependent footpad swelling....184

6.8. In vivo cytokine production in the footpads injected with normal or perforin-deficient Th1 or Th2 cells....185

6.9. Th1 and Tc1 cells produce similar levels of cytokines in vivo, while Th2 cells produce lower levels of cytokines than Tc2 cells, which correlates with the less footpad swelling they induced....186

Abbreviations

Ab	Antibody
AEC	3-amino-9-ethyl-carbazole
Ag	Antigen
APC	Antigen-presenting cell
BSA	Bovine serum albumin
CBH	Cutaneous basophil hypersensitivity
CFA	Complete Freund's adjuvant
CH	Contact hypersensitivity
Con A	Concanavalin A
CRBC	Chicken red blood cell
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
DTH	Delayed-type hypersensitivity
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorenscence-activated cell sorter
FBS	Fetal Bovine Serum
FIA	Freund's incomplete adjuvant
H-E staining	Hematoxylin and Eosin staining
HBSS	Hanks' balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin

ip	Intraperitoneal
ITS	Insulin-Transferrin-Sodium Selenite
iv	Intravenous
KLH	Keyhole limpet hemocyanin
LCMV	Lymphocytic choriomeningitis virus
LFA	Lymphocyte function-associated molecule
LT	Lymphotoxin (TNFβ)
MCP	Monocyte chemotactic protein
MIF	Macrophage migration inhibitory factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
PAGE	Polyacrylamide gel electrophoresis
PFA	Paraformaldehyde
PPD	Purified protein derivative
sc	Subcutaneous
SRBC	Sheep red blood cell
Tc	T cytotoxic
TGF	Transforming growth factor
Th	T helper
Thp	T helper precursor
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VLA	Very late activation antigen

Chapter I. Introduction

A. Prologue

The Ag-specific immune response is an important defense mechanism against a large range of invading microorganisms or non-self substances such as allergens and toxins. Based on the major components in the immune system that mediate the response, immune responses can be generally classified into two types, humoral immunity and cell-mediated immunity (also called cellular immunity). Humoral immunity is mediated by circulating antibodies (Abs) and can be transferred from an immunized to a naive individual by cell-free serum. Cellular immunity, on the other hand, is mainly mediated by T lymphocytes and can only be transferred by Ag-specific T cells. Humoral and cellular immunities are usually appropriate for controlling of different types of pathogens. Generally speaking, humoral immunity is more effective in clearing extracellular pathogens, while the cell-mediated immune response is more efficient in controlling intracellular infections. Thus, the choice of the type of immune response towards certain infectious microbes often determines the progress of the disease and the fate of the host. Humoral and cellular immunity against the same pathogen can be coexpressed in an individual, but during strong or chronic infections, these two types of immune responses are often mutually exclusive. Thus, understanding the regulation within each type, and between the two types of immune responses, can provide important information for manipulating our immune system to control diseases.

CD4 T helper (Th) cells are important regulatory cells required for both humoral and cellular immune responses. The immune regulatory functions of the Th cells are mainly mediated by their secreted cytokines. Effector Th cells produce heterogeneous patterns of cytokines and can be classified into distinct cytokine subsets. For the last ten years the classification of Th1 (producing IL-2, IFNγ and LT) and Th2 (synthesis of IL-4, IL-5, IL-6, IL-10 and IL-13) cells has been well established. In general, through their distinct cytokine profiles, Th1 and Th2 cells mainly regulate cellular and humoral immunity respectively. Furthermore, Th1 and Th2 cells express mutual cross-suppressive effects through some of their cytokines. More recently distinct cytokine patterns, similar to those of the Th1 and Th2 cells, have been identified among CD8 T cells, which are called Tc1 and Tc2 respectively. Tc1 and Tc2 cells showed similar in vitro activities, but their potential in vivo functions are still under investigation.

DTH is an important in vivo manifestation of cell-mediated immune responses which is particularly critical for controlling many intracellular infections. The ability to induce an Ag-specific DTH reaction often represents the reactivity of cellular immunity towards that Ag. Thus DTH provides an important model to study cell-mediated immune responses in vivo. DTH reaction is a cascade of events involving multiple types of cells and soluble factors. T cells are important regulators and effectors of the process. Both CD4 and CD8 T cells can induce DTH, depending on the MHC restriction of the Ags. Cytokines produced by T cells and other immunoactive cells are also important regulatory factors of DTH. In general, DTH is associated more often with Th1 than Th2 cytokines, although there are exceptions. The contribution of Th1 and Th2 cells and their cytokines to DTH responses is not fully understood.

Work reported here is mainly concerned with the regulation of DTH by CD4 or CD8 T subsets and their cytokines. Therefore in the first part of the introduction, I will present a brief review of different T cytokine subsets, the functions of their cytokines, and the potential physiological roles of the cytokine subsets during immune responses. In the second part of the introduction, I will give a brief summary of the general aspects of DTH and the cellular and molecular participants in DTH process.

B. T cytokine subtypes and their roles in immune regulation

Cytokines

The term cytokine was introduced (Cohen et al, 1979) to describe a group of regulatory polypeptides secreted by white blood cells and other nucleated cells. Up to now about 50 distinct cytokines have been identified, including the interleukins (ILs), the interferons (IFNs), the tumor necrosis factors (TNFs), the transforming growth factors (TGFs), the colony stimulating factors (CSFs) and many chemokines. Most cytokines are produced only transiently after cell activation. The same cytokine is usually produced by several unrelated cell types upon various stimuli, whereas one cell type is often able to secrete multiple cytokines after being activated.

Cytokines usually act over short distances as autocrine or paracrine intercellular signals. Released cytokines bind to high affinity receptors on the surface of target cells and lead to changes in proliferation rate, differentiation state, or expression of function in these cells. The effects of cytokines are often pleiotropic and redundant. One cytokine may exert different actions on different cells, while several structurally dissimilar cytokines can show remarkably similar actions on the same cell type.. Furthermore, functions of two cytokines can sometimes be synergistic or antagonistic, and one cytokine can stimulate or inhibit the synthesis of others. Thus, the numerous cytokine types, the pleiotropic functions of each cytokine and their complex interregulations make cytokines important regulatory mediators of immune responses.

General introduction of Th1 and Th2 cells

CD4 T helper (Th) cells are the major regulatory cells in all types of immune responses. Most of the immunoregulatory functions of Th cells are mediated through cytokines produced after the cells have been activated. Cytokine profiles of individual Th cells are usually heterogeneous, but not randomly distributed (Mosmann and Sad, 1996). By studying large numbers of mouse CD4 T-cell clones, Mosmann and colleagues have found that Th cells can be classified into at least two groups, based on their distinct cytokine patterns (Mosmann et al., 1986; Cherwinski et al., 1987). Type 1 Th (Th1) cells produce IL-2, IFNγ and lymphotoxin (LT) after activation through the T cell receptor, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Both Th1 and Th2 cells produce IL-3, TNFα, GM-CSF as well as many chemokines. Similar Th subsets have also been identified among human T cells isolated from chronically stimulated respondents (Romagnani, 1991a; Romagnani, 1991b). However, the production of IL-2, IL-6 and IL-10 in human Th cells is not as restricted to one subset as that of mouse Th cells. Functional studies of the Th1 and Th2 subsets show a general trend in the roles of these two types of cells in immune regulation (see below). Th1 cells normally activate cell-mediated immune responses such as DTH, while Th2 cells often induce humoral immunity and enhance allergy. The dichotomy of the immunoregulatory functions of Th1 and Th2 cells indicates the physiological importance of the Th1/Th2 classification.

Th1 and Th2 are not the only possible cytokine subsets among Th populations. Rather they represent two extreme phenotypes that correlate with certain strong immune responses (Kelso, 1995). Other cytokine producing subsets exist both in vitro and in vivo. For example, Th0 cells produce a mixture of Th1 and Th2 cytokines (Mosmann and Coffman, 1989). Furthermore, Th1 or Th2 cells are not homogenous within their populations. According to the single cell cytokine synthesis (Bucy et al., 1994), not all Th1 or Th2 cells produce the full range of their characteristic cytokine profiles, and the level of cytokine production by any single cell within the population varies. Nevertheless, the Th1 and Th2 distinct cytokine subsets provide us with a good system to study the class regulation of immune responses, particularly those associated with infectious diseases. Understanding of the regulation of immune responses can provide important information for manipulating the immune system to generate the most effective response for disease protection and cure.

The mechanisms of Th1 and Th2 cell differentiation have been extensively studied, but are not fully understood. Naive Th cells secrete only IL-2 when first stimulated by Ag/Ag presenting cell (APC). This type of cell is called T helper precursor (Thp) cells (Seder and Paul, 1994). Th1 and Th2 phenotypes can be generated from a single Thp cell under different differentiation conditions (Swain et al., 1990a; Sad and Mosmann, 1994). The pathway leading to Th1 and Th2 differentiation is still under investigation. There is evidence that the IL-4 gene is activated during Th1 cell differentiation, which suggests that the generation of Th1 cells may go through a intermediate Th0 phenotype (Kamogawa et al., 1993). However, whether Th1 cytokines are also produced during Th2 differentiation is not clear. The differentiation of Th1 or Th2 cells is influenced by multiple factors. From both in vitro and in vivo studies, cytokines are the most potent differentiation factors for generating Th1 and Th2 cells. IL-4 stimulates the generation of Th2 cells, while IFNγ, IL-12 and TGFβ each induce the development of Th1 cells (Seder and Paul, 1994). The choice to differentiate into Th1 or Th2 cells may also be affected by the costimulatory molecules on APCs, such as the B7 family (Keane-Myers and Nickell, 1995); and the dose of Ag (Hosken et al., 1995). During infections, differentiation of Th1 or Th2 cells is also controlled by the genetics of the host, hormone environments (Seder and Paul, 1994) and certain products of the pathogens (Hsieh et al., 1993). Thus, the generation of Th1 and Th2 cells is determined and regulated at different molecular and cellular levels.

Functions of Th1 and Th2 cytokines

Cytokines are one of the major mediators of the regulatory functions of Th cells. Thus, the activities of Th1 and Th2 cells during immune responses often correlate with the functions of their distinct cytokine profiles. In this section I will briefly review the biological functions of the characteristic cytokines of Th1 and Th2 cells.

The overall function of Th1 cytokines is to activate the cytotoxicity of multiple cell types, in order to mount an effective cell-mediated immune response. IL-2 is the principal growth and costimulatory factor for both CD4 and CD8 T cells (Smith, 1984; Erard et al., 1985). High doses of IL-2 result in the expansion of a potent cytolytic T cell population against tumor target cells (Rosenberg and Lotze, 1986). Similarly a high dose of IL-2 induces the proliferation, IFNγ production and cytolytic activity of NK cells (Ortaldo et al., 1984; Ortaldo et al., 1986; Trinchieri et al., 1984). IL-2 activated cytolytic cells (LAK cells) also provide a valuable tool in anti-tumor clinical practice. In addition, IL-2 also acts as a growth factor for activated human and murine B-blast cells, and increases Ig secretion (Miyawaki et al., 1987; Callard and Smith, 1988).

IFNγ is a potent activator for both macrophages (Le et al., 1983; Nathan et al., 1983) and neutrophils (Stevenhagen and van Furth, 1993), the major nonantigen-specific effectors of cellular immunity. IFNγ induces the production of TNFα and the reactive oxygen intermediates of macrophages (Philip and Epstein, 1986), and therefore activates the tumoricidal and antimicrobial activities of these cells. It also enhances the expression of both class I and class II MHC on macrophages (Sztein et al., 1984; Kelley and Pitha, 1985), which makes them better APCs for T cells. IFNγ increases IgG2a production by B cells (Snapper and Paul, 1987b) and the Fc receptor expression on macrophages (Warren and Vogel, 1985), and accordingly increases Ab-dependent macrophage phagocytosis and cytotoxicity. IFNγ also stimulates the expression of MHC class I Ag on virus infected cells and enhances CD8 T cell mediated cytotoxicity (Blackman and Morris, 1985; Bukowski and Welsh, 1985). All these results indicate that IFNγ is an important cytokine in cell mediated immune responses. Another Th1 cytokine, LT, in synergy with IFNγ, induces cytokine mediated cytotoxicity toward target cells (Lee et al., 1984). Overall Th1 cytokines stimulate the functions of macrophages, granulocytes, CTL and NK cells, the major participants in cell-mediated immune responses. Therefore, Th1 cells are most likely to activate cellular immunity such as DTH responses.

Th2 cells produce a number of factors that enhance Ab production and induce proliferation and activation of eosinophils and mast cells. IL-4 strongly increases the expression of class II MHC (Noelle et al., 1984), CD40 (Gordon et al., 1988; Valle et al., 1989) and BB1/B7 (Valle et al., 1991; Ranheim and Kipps, 1993) on B cells. These effects of IL-4 can enhance the cognate T-B-cell interaction and lead to increased Ab production. IL-4 also induces the clonal expansion of B cells activated by Ag (Alderson et al., 1987) and allows the proliferation of Ag-specific B cells in the presence of helper T cells (Stein et al., 1986). IL-4 causes the sustained proliferation of B cells activated through CD40 (Banchereau et al., 1991). During B cell differentiation, IL-4 regulates the Ab isotype switch to IgG1 and IgE (Rothman et al., 1988; Gauchat et al., 1992a; Gauchat et al., 1992b), the major isotypes in humoral and allergy responses. In IL-4 knock out mice, the production of IgG1 and IgE are strongly reduced (Kuhn et al., 1991), confirming the in vivo importance of IL-4 in generating these isotypes. IL-13, another Th2 cytokine, induces IgG4 and IgE synthesis independent of IL-4 in the human system (Punnonen et al., 1993). IL-5 also induces Ab production (Rasmussen et al., 1988) and B cell proliferation (Swain et al., 1988). But unlike IL-4, IL-5 acts at the late stage of B cell differentiation and induces IgM, IgG and IgA synthesis (Bond et al., 1987; Yokota et al., 1987) by selectively enhancing the proliferation of B cells bearing the above isotypes (Lebman and Coffman, 1988b). Furthermore, IL-5 synergizes with IL-4 to promote IgE secretion (Coffman et al., 1987) and enhances TGFβ-induced IgA production by LPS-stimulated B cells (Lebman et al., 1990). IL-10 also cooperates with TGFβ in inducing

IgA secretion by anti-CD40-activated human B cells (Defrance et al., 1992). IL-6 markedly enhances the production of all isotypes of Abs (Splawski et al., 1990) by B cells, and it appears to act at a late-stage of B-cell maturation (Muraguchi et al., 1988). Furthermore, IL-6 is especially crucial for IgE production driven by IL-4 (Vercelli et al., 1989). Overall, Th2 cells produce a cocktail of cytokines that activate B cells and enhance Ab production via different mechanisms.

Besides helping Ab production, Th2 cytokines also support the generation of mast cells, basophils and eosinophils. In the presence of IL-3, IL-4 induces the generation of both human basophils (Favre et al., 1990) and mouse mast cells (Rennick et al., 1987) from progenitor cells. IL-4 is an essential growth factor for the in vitro growth of connective tissue-type mast cells (Hamaguchi et al., 1987; Tsuji et al., 1990). IL-10 also enhances the proliferation of mast cell lines in synergy with IL-3 or IL-4 (Thompson Snipes et al., 1991). Furthermore IL-10 drives the differentiation of mast cells by inducing their protease MMCP1 and MMCP2 production (Ghildyal et al., 1992a; Ghildyal et al., 1992b). However, IL-10 inhibits the production of soluble factors such as histamine and TNFα from mast cells (Arock et al., 1996). IL-5 specifically induces the differentiation of eosinophils during hemopoiesis in vitro (Sanderson, 1992) and in vivo (Dent et al., 1990). In combination with IL-3, IL-4 induces the generation of eosinophils from human progenitor cells (Favre, et al., 1990). Both IL-4 (Tepper et al., 1989; Tepper et al., 1992) and IL-5 (Yamaguchi et al., 1988; Wang et al., 1989) are chemotactic factors for eosinophil homing to local tissue. IL-5 increases the expression of integrin CD11b on human eosinophils (Lopez et al., 1986), increases the adhesion of eosinophils to endothelial cells (Walsh et al., 1990), and thus may enhance the extravasation of these cells during inflammation. Together the above results indicate that Th2 cells have the potential to induce eosinophilia and mastocytosis in vivo. Furthermore, IL-5 has also been reported to modify basophil function and induce basophil differentiation (Bischoff et al., 1990; Denburg et al., 1991).

In vivo functions of Th1 and Th2 cells during immune responses

The in vivo functions of Th1 and Th2 cells are strongly influenced by the biological functions of their cytokines. In general, Th1 cells are often involved in cell-mediated inflammatory reactions. Th1 cytokines are commonly expressed at sites of DTH reactions (Tsicopoulos et al., 1992; Yamamura et al., 1991). Ag-specific Th1 cells are often isolated from individuals undergoing cell-mediated immune responses (Heinzel et al., 1991; Del Prete et al., 1994; Del Prete et al., 1991b; Haanen et al., 1991). On the other hand, Th2 cells are often found in association with strong Ab production and allergic responses (Mosmann and Sad, 1996). Th2 clones induce both B cell growth and IgE secretion (Lebman and Coffman, 1988a; Lebman and Coffman, 1988b), and the levels of Ig production are proportional to the number of Th2 cells added to B cells. Th2 phenotypes are often isolated from the sites of allergic responses (Van Reijsen et al., 1992; Robinson et al., 1992; Robinson et al., 1993).

The development of certain type of immune response can be either protective or deleterious to a given infection. Generally speaking, cell-mediated immune responses can control intracellular microorganisms, but may not be effective in clearing the extracellular pathogens and their secreted products. Ab responses are good at neutralizing free pathogens and their products, but are not efficient in controlling intracellular infections. The correlation between the Th subsets and the different types of immune responses in vivo (Mosmann and Sad, 1996) strongly indicates the crucial effects of Th1 and Th2 cells in immunoregulation.

The mouse *Leishmania major* infection model provides a clear demonstration of the immune regulation of Th1 and Th2 cells in disease progression. The control of this infectious disease requires the development of effective cell-mediated immunity capable of activating macrophages to a microbicidal state. Ab responses have weaker effect in clearing the intracellular organisms or in establishing immunity to reinfection. Choice of the type of immune response is dependent on the mouse strains (Scott et al., 1988; Locksley and Scott, 1991). Resistant mouse strains such as C57Bl/6, effectively localize the infection and are cured eventually. In these mice, macrophage activation and a strong DTH reaction develop after the infection, with only very low levels of Abs in the serum. Ag-specific T cells isolated from these animals show high IFNγ but low IL-4 production, suggesting a Th1 cell type (Locksley and Louis, 1992). In contrast, susceptible strains such as BALB/c, usually develop a severe and progressive disease that leads to death of the infected mice. These mice produce very high serum levels of Abs including IgE, but fail to mount *L. major* specific DTH reactions. T cells collected from these mice express high IL-4 but low IFNγ, which represents a Th2 phenotype (Locksley and Louis, 1992). The importance of the Th1 and Th2 cytokines to the disease progress has been confirmed by anti-cytokine Ab treatment. Anti-IFNγ Ab inhibited the cell-mediated immunity against *L. major* in the resistant strain and made these animals susceptible to the infection (Belosevic et al., 1989). On the other hand, anti-IL-4 treatment lowered Ab responses in susceptible mice and led to cure of the disease (Sadick et al., 1990). Direct evidence showing the distinct regulatory effects of Th1 and Th2 cells on disease progress has been demonstrated by adoptive transfer. When *L. major* specific Th1 and Th2 cell lines were transferred into *L. major* infected mice, Th1 cells transferred *L. major* specific DTH responses and provided protection for further infection, while Th2 cells exacerbated the course of the disease (Scott et al., 1988). Similar results have been obtained by transferring Th1 or Th2 cells into *L. major* infected SCID mice (Holaday et al., 1991). Effects of Th1 and Th2 cells in the transferred mice were mediated by IFNγ and IL-4 respectively. However as an exception, a *L. major* specific Th1 clone that was able to adoptively transfer parasite specific DTH exacerbated cutaneous leishmaniasis in infected naive mice. The mechanism of this action was not known (Titus et al., 1991).

Unlike the *L. major* infection, which is cured by a Th1 dominated cell-mediated immunity, the cure of mouse intestinal helminth infection is associated with a IL-4 dependent Th2 response (Locksley, 1994). In *Trichuris muris* infection, the nature of the immune responses towards the pathogen is also determined by the genetic backgrounds of the mice. Resistant mice such as BALB/k, expel adult worms and acquire protective immunity to rechallenge. Susceptible mice such as AKR, fail to clear the worms from the body and thus maintain a persistent infection (Else et al., 1994). These disparate outcomes of the infection are correlated with Th2 or Th1 cytokine profiles produced by mesenteric lymph node cells isolated from resistant and susceptible mice (Finkelman et al., 1991). The Th2 mediated response in resistant mice activates eosinophils, intestinal mastocytosis and IgE production. However, these hallmarks are not the critical mediators for host protection (Sher and Coffman, 1992), suggesting that some yet undetermined mechanisms related to Th2 responses may contribute to the resistance. Blocking of IL-4 results in persistent infection in the resistant mice, whereas neutralization of IFNγ allows the susceptible strains to clear worms (Urban et al., 1991; Urban et al., 1993). Thus, this system is another example demonstrating the dichotomous effects of Th1 and Th2 responses in infectious diseases.

Although direct tests, such as Th clone adoptive transfer or cytokine blockage, cannot be done in human systems, correlations between Th1/Th2 subsets and types of immune response also suggest distinct roles of Th1 and Th2 cells during immunoregulation. Th1 cells are normally associated with contact dermatitis, autoimmunity and some chronic inflammatory disorders. In contrast, Th2 cells appear to be involved in allergic responses against common environmental allergens and are responsible for triggering allergic atopic disorders (Romagnani, 1994). T cells isolated from the sites of tuberculin DTH reactions express high levels of IL-2 and IFNγ but low levels of IL-4 and IL-5, indicating a Th1 phenotype (Romagnani, 1991a). Similarly, nickel-specific T cell clones generated from individuals with nickel induced contact hypersensitivity, secrete IL-2 and high levels of IFNγ but low or undetectable levels of IL-4 and IL-5 (Kapsenberg et al., 1992). In contrast to DTH or contact hypersensitivity, atopic allergy is associated with elevated serum levels of allergen-specific IgE and local eosinophils. The allergen-specific Th cells from both peripheral blood and skin lesions of allergic patients showed elevated IL-4 and IL-5 production, but low levels of IL-2 and IFNγ, suggesting a Th2 phenotype (van der Heijden et al., 1991; Van Reijsen et al., 1992). T cells producing IL-4 and IL-5, but not IFNγ, are also found at the epithelial basement membrane in endobronchial mucosal biopsies (Hamid et al, 1991) and in the bronchoalveolar lavage fluid from patients with atopic asthma (Robinson et al., 1992; Robinson et al., 1993). Thus, similar to their mouse counterparts, human Th1 and Th2 cells are involved in the regulation of different types of immune reactions.

The dichotomy of Th1 and Th2 cytokines in immunoregulation has also been shown in many other animal or human diseases such as tuberculosis, leprosy, *Trypanosoma cruzi* and *T. equiperdium* infection, *Nippostrongylus brasiliensis* infection, and several autoimmune and allergic diseases (Mosmann and Sad, 1996). Based on these studies, a general trend of the physiological functions of Th1 and Th2 cells in immune regulation is demonstrated. Th1 cells generally mediate cellular immunity such as DTH, while Th2 cells are effective in supporting Ab production and enhancing allergy. However there are exceptions to this trend, e.g. the granulomas, chronic DTH responses, to the egg Ags of *Schistosoma mansoni* are caused by Th2 rather than Th1 cells (Wynn et al., 1993). The difference in regulatory mechanisms between Th1 and Th2 cell mediated DTH responses has not been fully understood, and will be further discussed in later sections.

Counter-regulations between Th1 and Th2 cells during immune regulation

Th1 and Th2 cells not only regulate different immune responses, but also mutually inhibit the functions of their reciprocal phenotypes through some of their characteristic cytokines. These cytokines inhibit the generation, the proliferation, or the biological functions of the reciprocal phenotypes. Furthermore, Th1 and Th2 cells often express opposite effects on non-T effector cells such as macrophages and B cells. This cross-regulation between Th1 and Th2 cells may partially explain the strong bias towards either Th1 or Th2 response during many infectious diseases (Mosmann and Sad, 1996), and the mutually exclusive phenomena between strong DTH and Ab responses (Parish, 1972; Katsura, 1977).

Th1 and Th2 cytokines mutually inhibit the generation and functions of the reciprocal phenotype. The Th1 cytokine, IFNγ, inhibits the proliferation of Th2 clones in response to IL-2, IL-4 or IL-1 (Gajewski and Fitch, 1988; Fernandez-Botran et al., 1988). The Th2 cytokine, IL-4, suppresses the development of Th1 cells by inhibition of IL-2 and IFNγ synthesis during T cell differentiation (Swain et al., 1990b). Another Th2 cytokine, IL-10, inhibits the cytokine productions of Th1 cells stimulated by macrophages (Fiorentino et al., 1989) and dendritic cells (Macatonia et al., 1993), and accordingly suppresses the biological functions of Th1 cells. The inhibitory effect of IL-10 is indirect, which acts through the inhibition of IL-12 production and the costimulator B-7 expression of the APCs (Murphy et al., 1994; Ding et al., 1993; Flores Villanueva et al., 1994; Chang et al., 1995). IL-10 also inhibits macrophage-stimulated Th1 cell proliferation, which is partially caused by the inhibition of synthesis of autocrine IL-2 from Th1 cells (de Waal Malefyt et al., 1991b; Ding and Shevach, 1992).

Th1 and Th2 cells often express opposite effects on other immunoactive cells, which further contributes to the inhibition of immune responses induced by the reciprocal phenotypes. Th1 and Th2 cytokines show different effects on macrophages and neutrophils. IFNγ is a potent activator for both macrophages and neutrophils, while some

Th2 cytokines show double edged effects on these cells. IL-4 (Crawford et al., 1988; Stuart et al., 1988) and IL-13 (McKenzie et al., 1993) are capable of inducing MHC expression on macrophages, which may enhance their ability to stimulate T cells. IL-4 increases the tumoricidal activity of murine peritoneal macrophages (Crawford et al., 1988) and it also acts on neutrophils to enhance their respiratory burst and their phagocytic properties (Boey et al., 1989), however the stimulating effects of IL-4 on macrophages and neutrophils are not as potent as that of IFNγ. On the other hand, IL-4 shows profound suppressive effects on macrophages. It downregulates expression of the three Fcγ receptors (Te Velde et al., 1990), and therefore blocks Ab-dependent cytotoxicity. IL-4 also inhibits the spontaneous and induced production of monokines, such as IL-1, IL-6, IL-8 and TNFα (Essner et al., 1989; Hart et al., 1989; Standiford et al., 1990; Miossec et al., 1992). IL-4 inhibits the synthesis of collagenase (Corcoran et al., 1992; Lacraz et al., 1992), superoxide (Abramson and Gallin, 1990; Ho et al., 1992), nitric oxide (Taub and Cox, 1995) and PGE_2 (Hart et al., 1989) of macrophages. These results suggest that IL-4 could be an anti-inflammatory factor.

IL-10 is a major suppressor of monocytes/macrophages and neutrophils. IL-10 inhibits the synthesis of several cytokines produced by LPS activated monocytes/macrophages, including IL-1, IL-6, IL-8, IL-10, IL-12, GM-CSF and TNF (de Waal Malefyt et al., 1991a; Fiorentino et al., 1991). The inhibition of its own production suggests a self-regulated expression pattern during immune responses. Besides cytokine production, IL-10 inhibits the microbicidal activity of macrophages during inflammatory responses. IL-10 reduces macrophage cytotoxic activity in synergy with IL-4 or TGFβ (Oswald et al., 1992a). By suppression of TNF synthesis, IL-10 reduces the TNF-mediated cytotoxicity of macrophages (Gazzinelli et al., 1992b; Oswald et al., 1992b), while the inhibition of nitric oxide synthesis (Cunha et al., 1992; Gazzinelli et al., 1992c) blocks another macrophage cytotoxic mechanism. Furthermore, IL-10 reduces the expression of class II MHC on certain types of macrophages (de Waal Malefyt et al., 1991b), thus, makes them poor APCs for T cells. IL-10 decreases the synthesis of IL-8 and Macrophage inflammatory protein (MIP) by neutrophils (Wang et al., 1994; Kasama et al., 1994). IL-10 also inhibits the production of IFNγ by NK cells (Hsu et al., 1992), partially through inhibition of the production of IL-12 (Germann, Rude and Mosmann, unpublished data), an NK cell stimulatory factor (Kobayashi et al., 1989; Chan et al., 1991). Thus, IL-10 is very likely to function as a potential anti-inflammatory reagent. However, IL-10 induces the expression of FcγR and increases cytotoxicity of human monocytes, which also suggests a potential role for IL-10 to enhance Ab-dependent cytotoxicity (Te Velde et al., 1992).

Th1 and Th2 cells also show different regulatory effects on B cell activation and Ab production. Th2 cytokines, in general, provide B cell help, and the degree of Ig production is proportional to the number of Th2 cells added to B cells (Snapper and Paul, 1987a; Del Prete et al., 1991; Del Prete et al., 1995). Th2 cytokines stimulate B cells to synthesize IgM, IgE and IgG1, but inhibit the production of IgG2a and IgG2b, while the Th1 cytokine, IFNγ, preferentially enhances IgG2a production but inhibits the production of IgG1 and IgE (Snapper and Paul, 1987b). IgG2a is very effective at fixing complement and promoting NK cell-mediated killing, so IFNγ can also enhance humoral immunities to destroy microbial pathogens. However, B cell help provided by Th1 cells shows a peak only at a T to B ratio of 1:1. At higher T:B ratios, the Ab production of B cells is suppressed (Coffman et al., 1988; Del Prete et al., 1991b). Furthermore, IFNγ blocks the responses of resting B cells to IL-4 (Rabin et al., 1986) and inhibits growth-stimulation and MHC class II enhancement of IL-4 on resting B cells (Mond et al., 1986). Collectively, these results suggest that Th1 cells can be both helpful and suppressive for B cells and their Ab production.

The mutual-inhibitory effects of Th1 and Th2 cytokines suggest a counter-regulatory modulation between polarized Th1/Th2 responses. There are evidences indicate the counter-regulation between Th1 and Th2 responses in vivo. During pregnancy, high levels of Th2 cytokines are expressed in the placenta, which may benefit fetal survival and placental maintenance (Lin et al., 1993). Interestingly, increased Th2 cytokine production correlated with the biased humoral and reduced cellular immunity often observed in maternal immune responses (Wegmann et al., 1993). Compared with nonpregnant mice, pregnant mice express higher Ab, but diminished DTH responses, against foreign Ags (Dresser, 1991; Holland et al., 1984). These phenomena suggested that the Th2 environment during pregnancy may be the cause of the enhanced Ab and suppressed DTH responses. More direct evidence provided recently showed that pregnancy impaired the Th1 immune response toward *L. major* in resistant C57BL mice (Krishnan et al., 1996). In pregnant C57BL mice, *L. major* infection became persistent correlated with reduced levels of IFNγ and TNF production upon *L. major* Ag stimulation.

The cross-regulation between Th1 and Th2 responses was also demonstrated in the mouse *S. mansoni* infection model. During the time of egg deposition, infected mice exhibited down-regulated IL-2 and IFNγ production toward parasite Ags, with a simultaneous increase in the production of Th2 cytokines (Pearce et al., 1991). Furthermore, these mice showed reduced Th1 and elevated Th2 responses to an unrelated foreign Ag (Kullberg et al., 1992). These results suggested that the elevated Th2 response towards schistosoma Ags may also alter cell-mediated immunity to other microbial agents. When the helminth infected mice were further infected with recombinant vaccinia virus, in contrast to control vaccinia-infected animals, the double infected mice failed to produce a detectable level of IFNγ toward the challenge by virus Ags, and virus clearance was delayed (Actor et al., 1993).

Both cell-mediated and humoral immune responses can be either protective or harmful to the host, depending on the pathogen. The counter-regulatory effects between the Th1 and Th2 cells and their cytokines provide the possibility to modulate immune responses during disease progression. Thus, determining which cytokines express mutual-inhibitory functions between Th1 and Th2 responses and understanding their functional mechanisms would provide potential therapeutical agents to regulate humoral or cell-mediated immune responses.

CD8 T subsets and their functions

CD8 T cells play an important role in anti-viral immunity, allo-graft rejection and anti-tumor responses. They are also of major importance in many bacterial and protozoan infections such as *Listeria monocytogenes, Mycobacterium tuberculosis, Toxoplasma gondii, Plasmodium spp, Theileria parvum, Leishmania major and Trypanosoma cruzi* (Scott and Kaufmann, 1991). The physiological functions of CD8 T cells are mainly mediated by either the cytolytic activities of these cells or by their secreted cytokines.

One of the major functions of CD8 T cells, also known as cytolytic T lymphocytes (CTL), is to mediate MHC class I restricted killing of infected or foreign target cells. The short-term cytotoxic activity of CD8 T cells is mainly mediated by two distinct mechanisms (Lowin et al., 1994; Henkart, 1994; Kagi et al., 1994b) depending on the target cells. Most of the target cells are killed by perforin-mediated pathway, which involves the regulated and polarized secretion of lytic granules, containing perforin and granzymes, upon contact of the T cell with a target (Liu et al., 1995). While for the Fas$^+$ target cells, they can also be killed by the Fas-mediated pathway, which involves the interaction of membrane-bound Fas ligand on T cells with the Fas molecule on the surface of the target cells (Rouvier et al., 1993). In both cases, CD8 T cells induce apoptosis of the target cells (Berke, 1995).

Like CD4 T cells, CD8 T cells also produce multiple cytokines when activated. In general, most CD8 T cells produce Th1 cytokines (Fong and Mosmann, 1990). However, IL-4 producing CD8 T cells from naive mice have been reported (Seder et al., 1992). High proportions of CD8 T cell clones derived from gut mucosa secreted IL-5 (Taguchi et al., 1990), and CD8 clones specific for *Mycobacterium leprae* Ags comprised both cytotoxic clones that produced IFNγ and suppresser clones producing IL-4 (Salgame et al., 1991). Recently distinct Th1- and Th2-cytokine patterns have been generated among CD8 T cells, which were named Tc1 and Tc2 cells respectively (Sad et al., 1995; Croft et al., 1994). Tc2-like cells were also isolated in vivo from microorganism-infected individuals (Coyle et al., 1995; Maggi et al., 1994; Paganelli et al., 1995) as well as normal human peritoneum (Birkhofer et al., 1996).

Allo-reactive Tc1 and Tc2 cells can be generated from naive CD8 T splenocytes in the presence of IL-12 with anti-IL-4 and IL-4 with anti-IFNγ respectively, and their cytokine phenotypes are not reversible (Sad et al., 1995). Cytokine regulation of Tc1 and Tc2 cells was studied in vitro. IL-2 was a potent growth factor for both Tc1 and Tc2 cells, while IL-4 also supported the growth of both subsets to a lesser extent (Pimlott and Mosmann unpublished data). IL-4 totally inhibited IL-2 production and reduced the level of other cytokines, such as IFNγ and LT, of mature Tc1 cells in an APC independent manner (Sad and Mosmann, 1995). These IL-4 treated Tc1 cells were then called cytokine-deficient Tc1 cells. TGFβ1 effectively inhibited the growth of both Tc1 and Tc2 cells. Unlike on CD4 Th2 cells, IFNγ did not inhibit the proliferation of either Tc1 or Tc2 cells (Pimlott and Mosmann unpublished data).

Since Th1 and Th2 cells usually exhibit different biological functions both in vitro and in vivo, functional differences were also expected from Tc1 and Tc2 cells. However the in vitro functions of allo-specific Tc1 and Tc2 cells were quite similar. They are both cytotoxic, mainly by the perforin-mediated pathway, with the Fas-mediated killing contributing to a lesser extent (Carter and Dutton, 1995). Neither Tc1 nor Tc2 cells provide cognate B cell help, probably due to their cytotoxicity to B cells, but Tc2 cells can provide bystander B cell help (Maggi et al., 1994; Sad et al., 1995). There are no obvious differences between Tc1 and the cytokine-deficient Tc1 cells in short term functional studies such as 4hr cytotoxicity assay and B cell help assay. But the cytokine-deficient Tc1 cells lost the ability for autonomous proliferation due to their loss of IL-2 production (Sad and Mosmann, 1995).

There is evidence for in vivo existence of Tc2 cells during infectious diseases. Some CD8 T cell clones (Maggi et al., 1994) derived from HIV infected patients with Job's-like syndrome produced high levels of IL-4 and IL-5, but no IFNγ. These cells expressed reduced cytolytic activity and provided bystander B cell help for IgE synthesis. In LCMV-specific TCR transgenic mice, a strong bystander Th2 response resulted in high levels of IL-5, but little IFNγ was produced by lung CD8 T cells. Airway eosinophilia developed in these mice when they were challenged intranasally with a LCMV-specific peptide (Coyle et al., 1995). These results indicate that Tc2 cells may be generated in vivo during a strong Th2 response, consistent with in vitro cytokine requirements for Tc2 differentiation. Furthermore, CD8 T cells producing IL-4 and IL-5 but not IFNγ were found in normal human peritoneum. These cells exhibited reduced cytolytic activity but provided substantial B cell help for IgG and IgA (Birkhofer et al., 1996). The generation and the in vivo biological roles of these cells need further investigation.

Like CD4 T cells, CD8 T cells can also mediate DTH reaction to MHC class I restricted antigens (Liew, 1982; Kundig et al., 1992). CD8 T cells are especially important in DTH toward infections by some viruses, such as LCMV (Doherty et al., 1990) and influenza virus (Leung and Ada, 1980), and other intracellular microbes, such as *Listeria monocytogenes* (Goossens et al., 1992), *Leishmania major* (Muller, 1992;

Muller et al., 1991), and *Cryptococcus neoformans* (Mody et al., 1994). In those infections, the DTH responses mediated by CD8 T cells either provide protection to the host, or are responsible for the pathogenesis of the disease. CD8 T cells are also the major mediators in certain contact hypersensitivity reactions (Bour et al., 1995; Xu et al., 1996). Furthermore, CD8 T cells can induce adoptively transferred footpad DTH (Lin and Askonas, 1981). However, the involvement and contribution of the CD8 T subsets with different cytokine secreting profiles to DTH responses is not known.

C. Delayed type hypersensitivity (DTH)

Concept, physiological significance and detection of DTH

The original concept of DTH came from the delayed skin reaction induced by tuberculin. A century ago, Robert Koch (1890) observed an induration and swelling at the local skin site after injecting tuberculin into previously sensitized individuals. This recalled skin reaction was first named the "Koch phenomenon". Later, similar skin reactions were induced by Ags from many other microbial organisms, e.g. bacteria, fungi, parasites and viruses, as well as by foreign proteins, tumors, allografts and simple chemical molecules (Turk, 1980). Generally, the reaction started a few hr after the Ag challenge as a white or rose colored swelling, and reached its peak at 24-72 hr. The skin reaction at its height was red and indurated, sometimes with hemorrhages at the center. The histological appearance was characterized by a perivascular accumulation of mononuclear cells, including lymphocytes and monocytes/macrophages. In mouse and rat, higher percentages of granulocyte infiltration also appeared (Boughton and Spector, 1963; Martins and Raffel, 1964, Turk, 1980). The ability to induce this reaction could be transferred to naive individuals by Ag-primed cells (T cells) but not by serum or Abs

(Chase, 1945, Zinsser, 1925, Zinsser and Mueller, 1925). This characteristic distinguished this reaction from immune responses caused by Ab-Ag complexes, such as the Arthus reaction. Collectively, this type of Ag-specific, T cell dependent, delayed skin recall reaction was later called delayed-type hypersensitivity or type IV hypersensitivity.

The physiological relevance of DTH has been explored extensively since the first discovery of this reaction. DTH is one of the major types of cell-mediated immunity, and can be both helpful and harmful to the body depending on the situation. The correlation between DTH and protection from many infections makes it a defined defense mechanism against a wide range of microorganisms, especially intracellular pathogens (Liew, 1982; Bochner and Schleimer, 1994). Whether or not a DTH is induced often becomes an important criterion in determining the effect of vaccines toward intracellular pathogens. Furthermore DTH skin test is widely used in estimating the prevalence of tuberculosis, histoplasma infection and coccidioisis infection in a given population, and the epidemiological information is valuable in directing-public health programs against these diseases (Ahmed and Blose, 1983). On the other hand pathogen induced DTH often causes tissue damage. In some cases it contributes to the major pathogenesis of the disease, as in lymphocytic choriomeningitis and tuberculosis (Crowle, 1975; Doherty et al., 1990). DTH is believed to be responsible for the destruction of self tissues in many autoimmune diseases, such as EAE (Turk, 1980), and to be one of the mechanisms that causes allograft rejection (Mason, 1988; Cramer, 1987). It has also been suggested that DTH participated in immunological surveillance and defense mechanisms against tumors. DTH induced by tumor-associated Ags or recall Ags often correlates with the clinical progress and the prognosis of tumors (Ahmed and Blose, 1983). Due to the involvement of DTH in a variety of immunological or immunophathological processes, understanding of this reaction is apparently important in the control of many diseases.

In human systems, DTH is tested by intradermal injection or epidermal painting of the Ags. Reactions are normally measured by the area of swelling (Ahmed and Blose, 1983). In animal studies, animals are immunized through a variety of routes such as cutaneous, subcutaneous, intradermal, epidermal etc. Very often the Ags are administrated with adjuvants to enhance the reaction (Crowle, 1975). DTH reactions are then induced by challenging the sensitized animals, often in footpads or ears, 1-2 wk after the immunizations. The magnitude of the DTH responses is recorded by the size of the tissue swelling, using either thickness or weight. Further studies of DTH include the histological study for cellular infiltration and the vascular permeability test for tissue edema. Infiltrated cells are often analyzed by dye or immunohistochemical staining. Vascular permeability is tested by iv injection of radio- or dye-labeled protein, and the radioactivity or content of dye in DTH tissue is then measured.

Classification of DTH responses:

DTH response is not simply one homologous type of reaction, rather it is characterized by considerable diversity. According to the nature of the Ags and the routes of immunization, the kinetics of DTH induction and expression, the induration and the histological profiles of DTH can be quite heterogeneous. Therefore, DTH responses are often subclassified into four types: The classical tuberculin type, Jones-Mote reaction, the granuloma and contact hypersensitivity.

The classical tuberculin DTH: Tuberculin DTH was so named to represent the type of skin reaction induced by tuberculin. It is the most common type of DTH induced in many infectious diseases. In animal studies, tuberculin DTH was induced by immunizing animals with Ags in complete Freund's adjuvant (CFA) containing mycobacteria. DTH was elicited 7 days after sensitization and inducibility was persistent for several weeks. This type of reaction was characterized by predominantly monocytic infiltration, sustained duration, and substantial fibrin deposition leading to marked induration (Dvorak et al., 1970; Turk, 1980). Striking microvascular alterations, basement membrane thickening and variable amounts of endothelial cell injury were also accompanied by the classical type of DTH reaction (Turk, 1980; Dvorak et al., 1980).

Jones-Mote Reaction: The concept of Jones-Mote reaction was introduced when it was found that the DTH reaction induced by soluble protein was not phenotypically similar to tuberculin DTH (Nelson and Boyden, 1964). In animal studies, Jones-Mote reaction was induced by immunizing animals with Ags in Freund's incomplete adjuvant (FIA) (Nelson, 1964), the Ag dose needed was much larger than that for classical DTH (Dvorak et al., 1980). The sensitizing time for DTH induction appeared earlier but was transient. The skin reaction was less persistent than that of classical DTH (Jones and Mote, 1934; Raffel and Newel, 1958), with little induration, probably due to less deposition of fibrin. Histologically, this reaction was characterized by granulocyte infiltration, especially basophils in guinea pigs. Therefore the Jones-Mote reaction was once called cutaneous basophil hypersensitivity (CBH) (Richerson et al., 1970). Basophil infiltration was suppressed when guinea pigs were treated with CFA before immunization (Nakamura et al., 1986). However, histological differences between tuberculin and Jones-Mote reactions were not absolute, and in many cases the type of infiltrates were quite similar in both reactions (Turk et al., 1980). Furthermore, T cells isolated from guinea pigs with a tuberculin DTH adoptively transferred Jones-Mote reaction in naive recipients (Askenase, 1976), suggesting that the cause of differences between tuberculin and Jones-Mote reactions may not be T cell dependent. However the mechanism that causes the differences between these two reactions is still not clear.

Granuloma: A granuloma is a localized collection of cells of the mononuclear phagocyte system (MPS) with or without other types of inflammatory cells. Granulomas are induced both non-immunologically by plastic beads, carbon particles, silica etc., and immunologically in tissues undergoing certain chronic infectious diseases such as tuberculosis, leprosy and schistosomiasis (Turk, 1980). Surrounding the central core of mononuclear phagocytes, there are usually lymphocyte, plasma cell, eosinophil and neutrophil infiltrations. Many of the mononuclear phagocytes show varying degrees of differentiation into epithelioid cells or giant cells. Central necrosis or caseation is often seen in a granuloma. Towards the periphery of the granuloma are varying degrees of fibroblast proliferation and collagen formation, which can lead to a site of intense fibrosis. Depending on the Ags, the T subsets that regulate the formation of the granuloma, and the histological appearance can be heterogeneous (Chensue et al., 1994; Chensue et al., 1995). Granulomas induced by Ags from schistosome eggs were mediated by Th2 cells, and the granulomas were largely infiltrated by eosinophils (Wynn et al., 1993). Endogenous Th1 cytokines inhibited both the size and the cellular infiltration of the schistosome granulomas (Chensue et al., 1995). Granulomas induced by the purified protein derivative (PPD) of *M. tuberculosis* on the other hand were mediated by Th1 cells and were mainly infiltrated by monocyte/macrophages (Chensue et al., 1995b) or CD8 T cells (Hernandez-Pando et al., 1995).

Contact hypersensitivity: Contact hypersensitivity (CH) is a delayed skin reaction induced by small chemical molecules instead of protein Ags (Eisen et al., 1952; Landsteiner and Jacobs, 1936; Chase et al., 1941). This reaction can be sensitized by repeated skin painting (Eisen et al., 1952) or multiple intradermal injections (Landsteiner and Jacobs, 1936; Chase et al., 1954). In vivo the chemicals covalently bind to skin proteins and create neoantigens (Eisen et al., 1952, 1958) to activate Ag-specific T cells (Moulon et al., 1995). CH is normally induced two weeks after the last sensitization. Histological appearance of CH is characterized almost solely by mononuclear cells with a low proportion (only 20-30%) of macrophages (50% in the tuberculin DTH) (Flax and Caulfield, 1963). The histological picture of CH is similar between human and guinea pig (Turk et al., 1966b). In general there are few polymorphonuclear cells at the site of reaction.

The process and the molecular and cellular participants of DTH:

DTH process: DTH is a cascade of cellular and molecular events expressed as a T cell dependent delayed inflammatory response. The whole DTH process can be arbitrarily divided into at least two sequential stages: the initiating phase and the effector phase. The initiating stage of a DTH response includes the generation of Ag-specific DTH-initiating T cells and the DTH effector T cells after the immunization, the activation of the endothelial cells and the recruitment of circulating DTH effector T cells after Ag challenge. The effector stage of DTH involves local activation of Ag-specific DTH effector T cells; synthesis of a cascade of chemotactic and activating cytokines from T cells, endothelial cells, and other infiltrating cells; further activation of endothelial cells; recruitment and activation of Ag-nonspecific monocytes/macrophages and neutrophils; increase of vascular permeability and deposition of fibrin (Askenase, 1992).

DTH initiating cells: DTH-initiating T cells, isolated from contact hypersensitivity reactions, were critical for the initiation of a DTH response (Askenase, 1992). These cells were Ag-specific, Thy-1$^+$, CD5$^+$, CD4$^-$, CD8$^-$, CD3$^-$, TCR$\alpha\beta^-$, TCR$\gamma\delta^-$, B220$^+$, IL-2R$^-$ and IL-3R$^+$, and were normally induced in the lymph nodes or spleen within one day after immunization. DTH-initiating cells function by releasing an Ag-specific DTH initiating-factor that binds to cutaneous mast cells in the tissue. After Ag challenge, binding of Ags to DTH-initiating factors on mast cells caused the release of serotonin from mast cells. Serotonin altered the integrity of endothelium, facilitated the rapid influx of mononuclear cells and triggered the effector stage of the contact hypersensitivity response. By adoptive transfer it was found that the Ag specificity and

MHC restriction of the DTH-initiating cells and DTH effector T cells did not need to be matched. Although the corresponding DTH-initiating cells have not been isolated from other DTH systems, mast cells and vasoactive amines were generally involved in all DTH initiation. This observation suggests that similar initiating procedures may occur in other types of DTH as well (Askenase, 1992). DTH could also be triggered by local tissue damage during the Ag challenge. The damaged tissue may send the so called "danger signals" through dendritic cells to the draining lymph node. This event induced the activation of Ag-specific T cells and caused cellular migration to the tissue (Matzinger, 1991). However the "danger signal" has to be further defined.

Vascular endothelial cells: vascular endothelial cells at sites of Ag challenge are critical players for both cellular infiltration and vascular permeability during a DTH reaction (Pober and Cotran, 1990a). Immediately after the initiation of inflammation, endothelial cells are activated by histamine or thrombin, etc., and later by cytokines. TNF is the major activating cytokine of vascular endothelial cells; other cytokines such as IL-1, IL-4, IFNγ and some chemokines are also able to activate endothelial cells. After activation endothelial cells produce cytokines (e.g. IL-6) and chemokines (e.g. IL-8 and RANTES), increase levels of existing adhesion molecules (e.g. ICAM-1) and induce expression of additional adhesion molecules (e.g. VCAM-1) on their surface (Pober and Cotran, 1990a). These changes slow the movement of circulating leukocytes, induce the attachment of these cells to the endothelium and enhance their extravasation. In addition, activated endothelial cells produce vasodilator substances such as prostacyclin (PCI_2) and nitric oxide (NO), which cause increased blood flow and optimize delivery of leukocytes to the site of inflammation. Endothelial cells also undergo shape changes and basement membrane remodeling, therefore increase vascular permeability and cause tissue edema. Plasma macromolecules such as fibrinogen also leak into the tissue. The deposition of fibrinogen and its insoluble cleavage product fibrin, as well as plasma fibronectin, serve as the basis of the induration often found in classical DTH (Geczy, 1984). Collectively, it has been demonstrated that vascular endothelial cells are active participants of DTH responses.

Adhesion molecules: Adhesive interactions, mediated by adhesion molecules expressed on both leukocytes and endothelial cells, are requisite for subsequent leukocyte extravasation at inflammatory sites (Anderson and Shaw, 1994). Three families of adhesion molecules are involved in this event: selectins, including L-selectin, E-selectin (ELAM-1) and P-selectin; the Ig superfamily, including intercellular adhesion molecule-1 (ICAM-1), ICAM-2 and vascular cell adhesion molecule-1 (VCAM-1); and the integrins, especially the β2 subfamily, lymphocyte function-associated molecule 1 (LFA-1), Mac-1, p150,95, and the very late activation antigen 4 (VLA-4) (Hogg, 1992). Integrins are mainly expressed on leukocytes (Springer, 1990). L-selectin is expressed on lymphocytes, while P- and E-selectin are found on endothelium. ICAM is found on both leukocytes and vascular endothelium, while VCAM-1 is restricted to endothelial cells. It has been hypothesized that emigration of circulating leukocytes into tissues involves sequential events: (a) initial rolling of leukocytes on inflamed vascular endothelium via selectin interaction with carbohydrate ligands on the endothelium; (b) activation of leukocytes by chemoattractants on glycoproteins on the surface of endothelium; (c) firm attachment to the blood vessel walls mediated by interactions between integrins and members of the Ig superfamily; (d) transendothelial migration (Shimizu et al., 1992). Activated or memory T cells express higher levels of adhesion molecules, indicating a selective migration of these cells to the inflammatory sites, although in an Ag independent manner (Anderson and Shaw, 1994).

During DTH or other inflammatory responses, infiltrates of varying cellular compositions are associated with the patterns of endothelial adhesion molecule expression (Silber et al., 1994). The expressions of E-selectin, ICAM-1 and VCAM-1 are induced or enhanced after the activation of endothelial cells. E-selectin mediated the adhesion of neutrophils and CD4 memory T cells to activated endothelium, and its expression was paralleled with neutrophil and lymphocyte infiltration (Silber et al., 1994). ICAM-1 induces T cell and monocyte/neutrophil infiltration by interacting with LFA-1 and Mac-1 respectively (Zimmerman et al., 1992). Blocking LFA-1 or ICAM-1 with Abs completely abrogated tissue swelling of Th2 cell mediated DTH, and the early granulocyte infiltration was inhibited by the Ab to ICAM-1 but not by that to LFA-1 (Muller et al., 1995a). Furthermore ICAM-1 deficient mice expressed diminished DTH (Xu et al., 1994). VCAM-1 mainly mediated the initial attachment of memory T cells through VLA-4, and anti-VLA-4 Ab blocked T cell migration to DTH sites (Issekutz, 1991; Elices et al., 1993). All these results suggest that the increased expression of these adhesion molecules on endothelial cells is responsible for the migration of different cell types. High levels of E-selectin were induced early (2-6 hr), while induction of VCAM-1 and ICAM-1 was delayed (12-48) (Pober and Cotran, 1990a). Sequential expression of adhesion molecules on the endothelial cell surface, at least partially controls the sequential dominant cellular infiltration seen in many DTH responses (Doherty et al., 1996).

Chemokines: The term chemokine is used to represent a group of low molecular weight (7-10 kD) proteins that express chemotactic activities for circulating leukocytes (Strieter et al., 1996). All the identified chemokines (about 30) are basic heparin-binding proteins structurally related by three highly conserved cysteine residues. Most chemokines are subdivided into two branches based on whether the first two cysteines in the motif are adjacent (termed C-C) or separated by an intervening residue (C-X-C). Recently a third subfamily containing one cysteine (-C-) was reported. In general, most of the C-C chemokines such as the monocyte chemotactic protein (MCP) family, RANTES, and the macrophage migration inhibitory factor (MIF) family, are chemotactic to monocytes and T cells, while the C-X-C chemokines such as IL-8 and platelet factor 4 (PF4), attract neutrophils (Schall, 1994). Chemokines are produced by multiple cell types including endothelial cells, T cells, macrophages, keratinocytes, fibroblasts etc. Secreted chemokines bind to endothelial cell surface heparin sulfate glycosaminoglycans, where they preferentially interact with leukocytes bound to endothelial cell through adhesion molecules. Chemokines express three separate actions on leukocytes. First, they increase the affinity of integrins on leukocytes to Ig superfamily adhesion molecules on the endothelium. Second, they change leukocyte spreading from a round immobile form to a flat migrating form. Last, they stimulate cell locomotion (Schall, 1994).

The expression and functions of some chemokines has been demonstrated in DTH responses. IL-8 (Larsen et al., 1995), γ interferon-induced protein (IP-10), MCP-1 (Kaplan et al., 1987; Gautam et al., 1994), RANTES (Devergne et al., 1994), macrophage chemotactic factor 1 (MCF 1) (Higashi et al., 1995) and MIF (Bernhagen et al., 1996) were elevated during classical DTH or contact hypersensitivity. These results suggest that these chemokines may be important participants in DTH responses. Furthermore, anti-IL-8 Ab suppressed the tissue swelling, and neutrophil infiltration of tuberculin DTH in rabbits (Larsen et al., 1995) and neutralizing anti-MIF Abs inhibited tuberculin DTH in mice (Bernhagen et al., 1996), which further confirms the important role of these chemokines in DTH. Local administration of IL-8 or zymosan-activated plasma (ZAP), a source of C5a, enhanced accumulation of large numbers of neutrophils and some lymphocytes in a PPD induced DTH sites in sheep (Colditz and Watson, 1992), suggesting their physiological roles in DTH. The production of chemokines during immune responses is often regulated by T cell derived cytokines. IFNγ induced the expression of IP-10 in macrophages and other cell types during DTH reaction (Kaplan et al., 1987). IFNγ and TNFα induced IL-8 and MCP-1 production from endothelial cells (Pober and Cotran, 1990a; Pober and Cotran, 1990b). IFNγ and TNFα induced, while IL-4 and IL-13 inhibited the production of the RANTES by endothelial cells (Marfaing-Koka et al., 1995). These results indicate a regulatory network of cytokines and chemokines in cellular infiltration during DTH responses.

T cells: T cells are both effectors and regulators of DTH responses. They not only control the Ag specificity of DTH, but also amplify the response by activating other Ag-nonspecific effector cells through a variety of cytokines and chemokines. Ag-specific T cells were generated in local lymph nodes (Cruz, 1994) stimulated by Langerhans cells or dendritic cells, the principal epidermal APCs that migrate to lymph nodes after Ag immunization (Bergstresser et al., 1991, Morikawa et al., 1992). Primed or memory T cells preferentially stay in the circulation and are able to transfer DTH responses in naive syngeneic animals. After skin Ag challenge, T cells are rapidly recruited in the site of Ag challenge where they react to the Ag, become activated, start proliferation and release cytokines. Both CD4 and CD8 cells migrated into the tissue of DTH responses (MacPhee et al., 1993; Gocinski and Tigelaar, 1990), but only less than 10% of the T cells were Ag-specific, while the vast majority of infiltrated cells were non-specifically recruited (Turk and Oort, 1963, Kalish, 1991). However, the efficiency of Ag-specific T cells in inducing DTH is high; it was demonstrated that under the right conditions, a single T cell is sufficient to locally transfer a footpad DTH response (Marchal et al., 1982). Activated T cells produced a group of cytokines and chemokines that recruited and activated Ag-nonspecific infiltrated effector cells (Pastore et al., 1996). Local or systemic administration of Ag after waning of the DTH still caused a flare-up reaction, indicating that some functional T cells still persisted at the inflammation site after DTH (Klasen et al., 1987).

DTH suppression: DTH is often under the negative regulation of suppressor cells. DTH suppressor cells can be T cells, B cells (Dorf and Benacerraf, 1984), or macrophages (Buchanan and Murphy, 1994). They function at either the induction or the effector stages of DTH, in either an Ag-specific or non-specific manner (Richard et al., 1990; Dorf and Benacerraf, 1984). DTH was often suppressed during strong humoral responses (Parish, 1972; Sher et al., 1983), or during certain infections (Liew, 1983; Richard et al., 1990; Nakamura et al., 1989). DTH suppression can also be induced by iv injection of a large dose of Ag (Dorf and Benacerraf, 1984) or by ultraviolet (UV) radiation of the animals (Denkins et al., 1989). The mechanisms of the DTH suppression were various. In certain cases, B cells and Ag-specific Abs mediated the suppression of DTH (Sher et al., 1983; Morikawa et al., 1989; Morikawa et al., 1990; Morikawa et al., 1991), which could partially explain the suppressed DTH during strong humoral responses. The mechanisms of DTH suppression by B cells and Abs are not clear. It was reported that some Ab suppressed DTH through idiotype-anti-idiotype interactions (Sy et al., 1979). CD8 T cells often serve as DTH suppressers. They function through their secreted Ag-specific suppressor factors that mediate suppressive activity at either the induction or effector phase of DTH (Ptak et al., 1986; Kuchroo et al., 1990). However the functional mechanisms of these suppressor factors are not well understood. It has been demonstrated that many suppressor factors contained two linked chains, one directly recognized the Ags while the other was I-J^+ that may control the genetic restriction (Dorf and Benacerraf, 1984). In addition, a few suppressor factors carry anti-idiotype specificity, and therefore might function via an idiotype-anti-idiotype interaction (Nash and Gell, 1981). UV radiation suppression of DTH was partially mediated through epidermal cells (Tamaki and Iijima, 1989). These cells inhibited DTH through direct production of suppressor cytokine. IL-10 and TNFα were the suppressor mediators for DTH and contact hypersensitivity respectively (Rivas and Ullrich, 1992; Brown et al., 1995). UV induced DTH suppression was also mediated by epidermal resident APCs that caused differentiation of Th2 cells instead of Th1 (Simon et al., 1990; Araneo et al., 1989) or functionally inactivated Th1 cells (Simon et al., 1994). The inhibition of DTH induced by Th2-like T suppressor cells was mediated by IL-4 and IL-10 (Rivas and Ullrich, 1994), while the inhibition of contact hypersensitivity was mediated either by IL-10 (Yagi et al., 1996) or by undetermined mechanisms (Rivas and Ullrich, 1994). Furthermore, in some cases, the impairment of the DTH response was due to a clonal deletion or anergy of DTH effector T cells (Thomsen and Marker, 1986; Marker and

Thomsen, 1986). Collectively, the wide existence of DTH suppressor cells and a variety of suppression mechanisms may explain the lack of DTH responses in certain systems.

Cytokines involvement in DTH: The general correlation between Th1 cells or Th1 cytokines to DTH reaction (Heinzel et al., 1989; Tsicopoulos et al., 1992; Pirmez et al., 1993) has been discussed previously. However, this correlation is not absolute. In certain cases both Th1 and Th2 cytokines were detected in DTH biopsies (Buchanan and Murphy, 1993; Ng et al., 1995), and the Ag-specific Th1, Th2 or Th0 cells were isolated from DTH sites (MacPhee et al., 1993; Picker et al., 1995). These results suggested that the micro-environment of DTH tissue was not always clearly Th1 biased. Furthermore, both IFNγ and IL-4 were elevated in the lymph nodes during initiation of oxazolone (OX) induced DTH, which suggested the involvement of both Th1 and Th2 cells in this type of DTH (Thomson et al., 1993), however, blocking Abs to neither IL-4 nor IFNγ altered the DTH swelling, suggesting that these two cytokines might not be the crucial mediators for the effector stage of the DTH reaction. The granulomas to the of *S. mansoni* egg Ags were caused by Th2 rather than Th1 cells (Wynn et al., 1993). Preactivated Th2 cells induced a DTH-like footpad inflammation that was inhibited by both anti-TNFα and anti-IL-4 Abs (Muller et al., 1993; Muller et al., 1994). These results suggest that Th2 cells also have the potential to induce DTH.

Direct functions of Th1 or Th2 cytokines in DTH responses have been studied by in vivo administration or by depletion of these cytokines. IL-2 given shortly after immunization suppressed, while given 4-10 days later enhanced SRBC induced DTH (Harada et al., 1986). Systemic injection of IL-2 during Ag challenge, but not immunization, increased the magnitude of dinitrochlorobenzene (DNCB) induced DTH, probably through the enhancement of T cell activity (Zaloom et al., 1991). Intradermal injection of IFNγ alone or in synergy with IL-1 recruited monocytes and lymphocytes into the skin, while anti-IFNγ inhibited lymphocyte recruitment to DTH sites (Issekutz et al., 1988a; Issekutz et al., 1988b). Anti-IFNγ also inhibited DTH induced by autoreactive T cells (Shiohara et al., 1988) and a variety of Th1 clones (Fong and Mosmann, 1989). All these results directly indicated the positive effects of Th1 cytokines on DTH. However herpes simplex viral Ag-specific DTH was induced in IFNγ-deficient mice with no significant difference from that in control mice (Bouley et al., 1995), suggesting that IFNγ was not absolutely required, at least for certain DTH reactions. Furthermore, anti-IFNγ administered during Ag challenge enhanced DNFP induced contact hypersensitivity in rats (Skoglund et al., 1988), and IFNγ administration prior to challenge reduced the reaction (Skoglund and Scheynius, 1990), suggesting that IFNγ could also be a negative factor in DTH induction. IL-4, through its action on γδ T cells, played a critical role in T cell migration in a systemically transferred DTH response (Salerno et al., 1995). Furthermore, anti-IL-4 treatment not only suppressed the DTH induced by trinitrochlorobenzene (TNCB), but also blocked the ability of T cells to transfer DTH to naive mice (Dieli et al., 1994). These results indicate that IL-4 plays a crucial role, at least in certain types of DTH.

Besides Th1 and Th2 specific cytokines, inflammatory cytokines such as IL-1, IL-6 and TNFα, which are produced by many cell types, are actively involved in DTH responses (Chu et al., 1992, Pastore et al., 1996). TNFα is the most potent activator of vascular endothelial cells (Pober and Cotran, 1990a; Pober and Cotran, 1990b). In addition, TNFα recruited lymphocytes to local skin in a dose-dependent manner (Kalaaji et al., 1989). Furthermore, both IL-6 and TNFα showed augmented effects to DTH responses (Jayaraman et al., 1990). However, in vivo administration of TNFα inhibited DTH, probably due to its suppression effects on T cells (Gordon and Wofsy, 1990). The physiological functions of TNFα during inflammation also depended on local cytokine profiles of T cells. In a Th1 cytokine environment it acted as an additional macrophage-activating factor, while in mixed Th1 and Th2 (or Th0) responses it caused more severe tissue damage (Hernandez-Pando and Rook, 1994). Although IL-6 was elevated during

DTH responses (Chu et al., 1992), in vivo administration of IL-6 inhibited both the induction and the effector phases of SRBC induced DTH (Mihara et al., 1991). IL-1 was elevated during DTH responses, but IL-1β-deficient mice developed normal DTH, suggesting that IL-1β was not an essential mediator for DTH (Zheng et al., 1995). TGFβ1 inhibited the initiation and the effector stages of SRBC-induced DTH (Meade et al., 1992). An IL-10-containing keratinocyte supernatant inhibited the initiation of DTH against alloantigens (Rivas and Ullrich, 1994). All these results demonstrate that DTH is regulated both positively and negatively by multiple cytokines derived from a variety of cell types. IL-12 may be a positive regulator for DTH by promoting Th1 functions. Macrophage migration inhibitory factor (MIF), the first cytokine found in DTH responses is crucial for DTH induced by tuberculin (Bernhagen et al., 1996)

Granulocytes: Granulocytes are important nonspecific DTH effector cells and are the earliest infiltrates at DTH sites. The degree of neutrophil infiltration was directly correlated with the intensity of both DTH and CH (Lee et al., 1988), and proportional to the degree of hemorrhagic necrosis of the dermis (Weissmann, 1989). Deletion of neutrophils inhibited both the priming and effector phases of DTH, and the recruitment of monocytes and T cells (Kudo et al., 1993a; Kudo et al., 1993b). Direct injection of neutrophils or neutrophil protease induced monocyte infiltration and tissue swelling similar to Ag-induced DTH or CH (Lee et al., 1988). All these results suggest the contribution of neutrophils in the inflammatory process of DTH. Besides neutrophils, other granulocytes are also involved in DTH. Although eosinophils are normally dominant in Th2 responses, significant numbers of activated eosinophils were isolated at human DTH sites (Gaga et al., 1991). But the role of eosinophils in DTH responses is not clear. Basophils are the major infiltrate of Jones-Mote DTH in guinea pigs (Richerson et al., 1970). In human DTH reactions, basophils were related to the early phase of DTH (Chavance et al., 1991). Mast cells were critical for initiating vasoactivation in DTH responses (Van Loveren et al., 1983), T cell recruitment

(Askenase et al., 1983) and the infiltration of other inflammatory cells (Herrmann et al., 1988). Therefore, these cells are also active participants in DTH.

Monocyte/macrophages: The recruitment and local activation of macrophages is often considered the hallmark of DTH responses. Perivenous mononuclear cell migration begins as early as 2 hr after Ag injection, and progressively increases to a maximum at about 48 hr (Turk et al., 1966a). Infiltrated macrophages were primed by T cell derived cytokines, mainly IFNγ but also GM-CSF and IL-4, to become cells ready to be activated (Adams and Hanna, 1984). The primed macrophages were then activated by bacterial products or lymphokines, such as LT, TNFα and IL-1, or by tumor cells. The activated macrophages increased their capacity to effect cytotoxicity against a broad range of neoplastic and infectious targets, and are major players in expressing the biological functions of DTH responses. The cytotoxic activity of macrophages increases within 4 hr after being activated, and reaches maximal levels by 8 to 12 hr, then progressively decreases to baseline by 24 hr. The loss of the cytotoxicity is irreversible, which might be one of the mechanisms that quenches the DTH response after 24-48 hr. The activated macrophages also result in activation of the coagulation cascade and the fibrinolytic pathways causing deposition of fibrin (Ahmed and Blose, 1983; Geczy, 1984). The insoluble deposits of fibrin trap both proteins and fluids and assume the structure of a gel. It is the local fibrin gel formation rather than the accumulation of mononuclear cells that is responsible for both edema and induration of DTH responses.

Summary: In a very brief summary, the DTH response is a multi-step process with the participation of many cell types and soluble factors. DTH initiating cells sensitize the tissue for an obligatory initial vasoactivation, which allows DTH effector T cells and other Ag-nonspecific effectors to enter the local tissue. Ag-specific T cells are activated in situ, producing a variety of cytokines and chemokines. These soluble factors further activate the vascular endothelial cells and the infiltrated macrophages and granulocytes, which express the biological functions of DTH, such as controlling and clearing pathogens or other foreign substances.

Adoptively transferred DTH

DTH response can be transferred from a sensitized individual to a naive individual by T cells (Zinsser, 1925, Klasen et al., 1987). This characteristic of DTH not only demonstrates the T cell dependence of this response, but also provides an important model to study DTH. The Ag specificity, the MHC restriction of DTH (Miller et al., 1975; Miller et al., 1976), and more recently, the contribution of CD4 T cytokine subsets to DTH responses were all investigated using adoptively transferred DTH models (Cher and Mosmann, 1987). The systemically and locally transferred DTH are two commonly used DTH models.

Systemically transferred DTH: This DTH is induced by injecting Ag-specific T cells intravenously or intraperitoneally into naive animals, then challenging the animal's skin with the corresponding Ags. A skin swelling occurred at the site of Ag challenge which appeared similar to the DTH induced in the donor animals (Chase, 1945; Stavitsky, 1948; Kirchheimer et al., 1949). This process bypassed the stage of Ag-specific T cell generation, and made the naive recipients similar to primed individuals.

The fate of transferred cells in the recipient has been studied using radio-labeling. The majority (around 80%) of the cells were trapped in the liver and lung 10 min. after transfusion, and only less than 4% of the injected cells were remained in the circulation (Turk, 1962). These results suggested that adoptively transferred DTH was induced by only a small fraction of the injected T cells. The proportion of labeled cells in the lesion was related to the proportion of labeled cells in the peripheral blood. More than 90% of the infiltrating cells were of recipient origin and injected cells did not make a significant contribution to the cellular infiltrate (Turk and Oort, 1963). Furthermore, there were similar amounts of labeled cells in Ag challenged sites and control sites. However, using an Ag-specific T cell clone, it was found that there were more clone cells infiltrated at the Ag challenged DTH sties than control sites (Sano and Shimao, 1993), which could be due to retention or further recruitment of the Ag-specific cells by the ongoing inflammation or local T cell proliferation. This result demonstrated that local cellular recruitment was not Ag dependent, but it was the activation of the Ag-specific T cells that caused the lesion induced by the specific Ags significantly larger than that induced by control Ags.

When using pure Ag-specific T cells or clones, it was found that T cells alone may not be sufficient to induce systemically transferred DTH, at least in certain cases (Cher and Mosmann, 1987; Dieli et al., 1994). These observations suggested that other Ag-specific signals or cells were required for DTH responses. As previously discussed, DTH initiating T cells were required to initiate the DTH reaction in the systemically transferred DTH process (Askenase, 1992). Recently another study reported that IL-4, through its action on γδ T cells, was required for Ag-specific αβ T cells to systemically transfer contact hypersensitivity (Dieli et al., 1994). However the mechanism of the IL-4 and γδ T cell functions in DTH is not clear.

Locally transferred DTH: Locally transferred DTH is induced by injecting primed T cells together with their corresponding Ags intradermally or subcutanously into naive animals (Bianchi et al., 1981; Scovern and Kantor, 1982). This type of DTH presented a similar time course and histological appearance to DTH induced by direct Ag challenge of primed animals. The reaction represents only the effector stage of the DTH process, since T cell generation and recruitment are not required. Besides primed T cells and their Ags, this reaction can also be transferred by activated syngeneic T cells (Muller et al., 1993), naive αβ TCR transgenic T cells with Ag (Sato et al., 1994) and activated xenogeneic T cells (Trial, 1989). These results demonstrate the importance of the T cell derived signals to the DTH reaction. In the locally transferred tuberculin DTH, the reaction was detectable as early as 4hr after T cell and Ag injection, with erythema, induration and often hemorrhage. Blood vessels at a distance from the injection site were surrounded with a dense cuff of mononuclear cells, which was not seen in control sites injected with T cells without Ags, or nonrelevant Ags (Metaxas and Metaxas-Buhler, 1955, Waksman and Matoltsy, 1958). There was a marked polymorphonuclear infiltration around the center of injection site. The polymorphonuclear infiltrate between test and control sites was quantitative rather than qualitative, but the perivascular infiltrate observed around peripheral vessels at 4 hr after the T cell injection was not present in the control sites (Blazkovec et al., 1965, Turk and Polak, 1967), therefore it is a hallmark for the locally transferred DTH.

The locally transferred DTH model provides a useful system to study the potential contribution of different T subsets to DTH reaction. Resting Th1 but not Th2 T cell clones induced DTH when injected together with their Ags into the footpads of naive mice (Cher and Mosmann, 1987). This reaction was dominated by neutrophil infiltration and was partially inhibited by anti-IFNγ (Fong and Mosmann, 1989), which agreed well with the general correlation between Th1, but not Th2 cells, and DTH responses. However, later it was found that not all Th1 clones induced footpad DTH (Wong et al., 1988). Furthermore Th2 cells, when pre-activated, also induced a DTH-like footpad inflammatory responses (Muller et al., 1993). The Th2 mediated reaction was accompanied by both neutrophil and macrophage infiltration, but unlike the footpad DTH induced by Th1 cells, this reaction was IL-4-dependent (Muller et al., 1993). These results suggest that Th1 cells are more active in DTH induction, while Th2 cells also have potential to induce DTH-like inflammation through different mechanisms. Why Th2 cells and their cytokines are not normally associated with DTH responses is not currently understood.

Chapter II. Rationale and objectives

A. Rationale:

DTH is an important type of cell-mediated immune response crucial in controlling many intracellular infections. T cells are important participants and regulators of DTH response. Functions of T cells are often mediated by their secreted cytokines that express various effects on other cell types. Studies based on large numbers of human or mouse diseases have shown that DTH is often associated with Th1 cells and their cytokines. These results suggest that Th1 cells and their cytokines are the activators or positive regulators of DTH responses. However, it was reported that in certain cases, Th2 cells are also involved in DTH-like inflammation or granulomas. This indicates that Th2 cells also have the potential to mediate DTH. But the mechanism of Th2 cell inducing a DTH response has not been fully understood. Studies of the contribution of different T subsets and their cytokines to the DTH reaction are important for understanding this response. The physiological function of DTH is mediated at the effector stage of the reaction through the actions of multiple cell types and their soluble factors. DTH effector T cells and their cytokines function mainly at the effector phase of the reaction in local tissues. Therefore the local adoptively transferred DTH model, which represents the effector stage of DTH after the event of T cell migration, was used to study the contributions of CD4 or CD8 T subsets and their cytokines to DTH reaction.

In vitro, Th1 and Th2 cells are often negatively cross-regulated through their cytokines. This suggests that the same cytokines may also cross-regulate the immune responses induced by the reciprocal phenotypes. Therefore some of the Th2 cytokines may inhibit Th1 cell induced DTH. IL-10 produced by Th2 cells and macrophages inhibits the activation, cytokine production, and proliferation of Th1 cells, It also inhibits monokine productions and microbicidal activity of macrophages, as well as chemokine productions of neutrophils. Furthermore, in vivo IL-10 expression is often associated with increased Th2 type of responses and suppressed Th1 type of responses. All these results suggests that IL-10 may inhibit Th1 cell induced DTH. Using mouse recombinant IL-10, I tested the direct effects of IL-10 on the effector stage of DTH responses induced by Th1 cells. We believe that the results from this study not only provide information on cytokine regulation of DTH, but also help to search for therapeutical agents for DTH inhibition, since DTH can also cause serious tissue damage and be harmful to the host in certain diseases.

Although numerous studies have investigated the potential role of Th1 or Th2 cells in DTH responses, little is known about whether the different cytokine profiles would affect the ability of CD8 T cells to induce DTH. CD8 T cells are important effectors and regulators in DTH responses, especially in certain virus and intracellular pathogen infections. Both the bulk CD8 population and CD8 T cell clones were also able to adoptively transfer DTH in naive animals. Recently subsets of CD8 T cells with distinct cytokine patterns (Tc1 and Tc2 cells) were generated in vitro and isolated in vivo. Therefore, the potential in vivo functional difference of these two CD8 cytokine subsets needs to be elucidated. Since resting Th1 and Th2 clones show different abilities to locally transfer DTH in naive mice, the contribution of Tc1 and Tc2 cells in DTH responses was studied using the locally transferred DTH model. Furthermore, the characteristics and cytokine regulation of DTH induced by Tc1 and Tc2 cells were investigated.

Rapid cytotoxicity mediated by perforin is one of the major biological functions of CD8 T cells, and sometimes it is also expressed by CD4 T cells (see chapter VI). However whether the perforin-mediated cytotoxicity contributes to the DTH responses induced by different T subsets is not clear. Among the in vitro generated allo-reactive T cells, both Tc1 and Tc2 cells are highly cytolytic mainly by the perforin pathway, and Th1 cells also showed moderate perforin-mediated killing. The rapid killing of certain target cells in vivo by these allo-reactive T subsets could be a positive factor to the DTH reaction, since dying cells may release pro-inflammatory or other signals to initiate an inflammation. The generation of perforin-deficient mice provides a unique system to test the involvement of perforin in DTH reactions. Therefore, using Tc1 and Tc2, and also Th1 and Th2 cells generated from perforin-deficient mice, the involvement of perforin-mediated cytotoxicity in DTH responses was tested.

B. Objectives:

A. Purify a large amount of recombinant mouse IL-10 expressed by mammalian cells, test the effects of IL-10 on Th1 clone adoptively transferred DTH, and the effector stage of DTH induced by sheep red blood cells (SRBCs), and address the mechanisms of the IL-10 effects on DTH.

B. Generate alloreactive Tc1 and Tc2 cells in vitro; test the potential abilities of these cells to adoptively transfer footpad DTH responses in naive mice bearing target MHC molecules; and study the edema, cellular infiltration and the local cytokine environment of the DTH responses induced by Tc1 or Tc2 cells.

C. Generate alloreactive Th1, Th2, Tc1 and Tc2 cells from perforin-deficient mice, test and compare the abilities of these cells to adoptively transfer DTH in naive mice bearing target MHC, and test the in vivo activation of these cells.

Chapter III. Materials and methods

A. Materials and Methods (General)

Animals

Female BALB/c mice, 8-12 weeks old were purchased from Health Sciences Laboratory Animal Services (HSLAS, University of Alberta). Female C57BL/6, F1 mice of BALB/c x C57BL/6, and the SCID mice, 8-12 weeks of age, were obtained from the Jackson Laboratories, Bar Harbor, Maine. The perforin-deficient mice were kindly provided by Dr. D. Kagi (Toronto, Canada) and Dr. H. Hengarnter (Zurich, Switzerland). The mice were maintained in Health Sciences Laboratory Animal Services, Edmonton, Alberta, Canada, in accordance with the guidelines of the Canadian Council on Animal care.

T cell clones and cell lines

JE 9-2 cell line was kindly provided by Dr. J. F. Elliott. (detail see chapter IV). M264-15 is an A.TL-derived allospecific Th1 clone specific for H-2^d MHC (Cher and Mosmann, 1987). HDK-1 is a BALB/c-derived Th1 clone specific for a soluble antigen, keyhole limpet hemocyanin (KLH) (Cher and Mosmann, 1987). MD13-5.1 and MD13-10 are BALB/c-derived Th1 clones specific for chicken red blood cells (CRBC) (Mosmann et al., 1986). WEHI 164.13 is a TNF/LT-sensitive cell line (Torres et al., 1982). M12.4.1 is a B lymphoma expressing H-2^d, and was obtained from Dr. L. Glimcher (Glimcher et al., 1983). J774 is a macrophage cell line expressing H-2^d, and was obtained from Dr. M. Belosevic (Edmonton, Canada). Sheep red blood cells (SRBC) and Chicken red blood cells (CRBC) were purchased from Triage Microbiologicals (Ardrossan, Alberta, Canada).

Reagents

Cytokines:

Mouse recombinant IL-2 was expressed in *Escherichia coli* (Zurawski et al, 1986) and used as a partially purified preparation. Recombinant IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF and IL-12 were expressed in COS cells (Lee et al., 1986), and used as a dilution of COS transfected supernatants. JE 9-2 IL-10 was expressed by a BW5147 mouse lymphoma cell line transfected with mouse IL-10 cDNA (Li et al., 1994).

Antibodies:

Anti-IFNγ and anti-IL-4 antibodies were purified from the supernatant of XMG1.2 (Cherwinski et al., 1987) and 11B11 (Ohara and Paul, 1985) hybridomas respectively, using a protein G column (Sigma, Mississauga, Ontario). Rat-anti-mouse Gr-1 (IgG2b), CD4, Ly-2-FITC (anti-CD8a, clone YTS 169.4) and L3T4-PE (clone YTS 191.1) monoclonal antibodies were purchased from Cedarlane Laboratories Ltd. (Hornby, Ontario). Rat-anti-mouse Mac-3 (IgG1), Ly3-PE (anti-CD8b, clone 53-5.8), NK-1.1-PE (clone PK136) and CD44-FITC (clone IM7) monoclonal antibodies were bought from Pharmingen (San Diego, CA).

Others:

Methotrexate (MTX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Insulin-Transferrin-Sodium Selenite (ITS) serum-free medium, 3-amino-9-ethyl-carbazole (AEC), 2,2'-azino-bis(3-Ethylbenz-Thiazoline-6-Sulfonic acid) (ABTS), Bovine serum albumin (BSA), collagenase I, DNase, Evans Blue and paraformaldehyde (PFA) were purchased from Sigma Chemical Co., (St Louis, MO). Fetal Bovine Serum (FBS) was obtained from Hyclone Labs (Logan, Utah). RPMI 1640, Hanks' balanced salt solution (HBSS) and CFA were from GIBCO (Grand Island, NY).

The Lympholyte-M CL5030 ficoll, was purchased from Cedarlane Laboratories Limited. Peroxidase-conjugated streptavidin was bought from Jackson (West Grove, PA).

Cytokine ELISA

Most cytokines reported in this thesis were measured by double mAb sandwich enzyme-linked immunosorbent assays (ELISA). First and second antibodies for each cytokine were: IL-2, JES6-1A12 and JES6-5H4-biotin (Pharmingen, San Diego, CA, USA); IL-4, BVD4-1D11 and BVD6-24G2-biotin (PharMingen); IL-5, TRFK5 and TRFk4-biotin (PharMingen); IL6, MP5-20F3 and MP5-32C11 (Pharmingen); IL-10, SXC1 and SXC2 (Mosmann et al., 1990); IFNγ, R4B6 (ATCC) and XMG1.2 (Cherwinski et al., 1987). For all the cytokine assays, PBS was used as the coating Ab buffer, while PBS with 1% BSA and 0.02% Tween 20 was used as secondary Ab and streptavidin dilutent. ABTS was used as the substrate. All incubation steps were done at 37°C for 30 min. The sensitivities of those assays are: IL-2, 0.06 ng/ml; IL-4, 0.6 ng/ml; IL-5, 0.6 ng/ml; IL-6, 0.6 U/ml; IL-10, 1.2 ng/ml; and IFNγ, 0.6 ng/ml.

Cytokine bioassays

The IL-10 inhibition of IFNγ production by IL-2-stimulated mouse spleen cells was measured to determine the IL-10 biological activity. 10^6 spleen cells were cultured with 10 ng/ml of mouse recombinant IL-2 and different concentrations of IL10 in a total volume of 200µl. Plates were incubated at 37°C for 36 hr, and the supernatants were assayed for IFNγ by ELISA. The inhibition of IFNγ production by IL10 samples was compared with inhibition by an IL10 standard, and units of IL10 biological activity were calculated. The bioactivity of LT/TNF was tested as WEHI 164.13 cytotoxicity using MTT assay as previously described (Lewis et al., 1986). The sensitivity of TNF bioassay is 0.01 pg/ml.

Measurement of footpad swelling

Footpad DTH was induced by different cells according to the experiments (see materials and methods specific for the later chapters). Footpad swelling was measured using a Mitutoyo gauge (NO.7312, Mitutoyo, Elk Grove Village, IL, USA). Each number of footpad swelling was an average of three sequential measurements. Occasionally a quality control experiment was performed to check the consistency of the measurement. Each mouse was placed in a separate cage, the footpad swelling was measured and recorded in a card, the card was folded and kept in the corresponding cage. The cages were then rearranged and another round of measurement was performed. Normally three or four rounds of measurements were done, and the mean and standard deviation (SD) were calculated. Figure 3.1 shows the results of one representative experiment. In most experiments, the animals were coded and measured blind. Blind and non-blind experiments yielded similar results.

B. Materials and Methods Specific for Chapter IV

Recombinant IL10 purification

JE 9-2 cells were cultured in 8% FBS in RPMI 1640 with 0.5μM MTX and then transferred to ITS serum free medium at a density of 10^6 cells/ml and cultured for 3 days. The supernatant was then concentrated about 100-fold by ultrafiltration using an Amicon YM3 membrane (Amicon, Danvers, MA, USA). The concentrated supernatant was passed through a Blue 3GA reactive dye column (Sigma), the column was washed with 10mM Tris-HCl, pH 7.6, and IL10 was eluted with 10mM Tris-HCl, 1.5 M NaCl, pH 7.6. All the fractions were tested for IL10 by ELISA, the positive fractions were pooled, concentrated using an Amicon YM3 membrane and dialyzed overnight against 20mM Tris-HCl, pH 8.6. This material was further purified on a Mono Q HR 10/10 ion exchange column (Pharmacia, Baie D'Urfe, Quebec, Canada). Elution was carried out with a linear salt gradient of NaCl from 0 to 0.2M in 20mM Tris-HCl, pH 8.6. The IL10-positive fractions, identified by ELISA, were concentrated and dialyzed overnight in PBS. The purified IL10 was stable at -70°C for at least a year. The protein concentration of purified IL10 was tested by Bio-Rad protein assay (Bio-Rad, Ontario, Canada), or determined by the extinction coefficient (1 mg/ml = 0.36 A_{280}) (Howard et al., 1993). The endotoxin level in the purified IL10 was tested using an endotoxin test kit (Sigma), and all the batches used in the experiments reported here contained <0.5ng endotoxin/100μg of IL10. Intraperitoneal injection of 1.5ng LPS per mouse had no effect on M264-15-induced footpad swelling.

IL10 western blot

IL10-containing samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel. Proteins on the gel were transferred onto a nitrocellulose membrane (MSI, Westboro, MA, USA) using an electrophoretic transfer cell (BIO-RAD, Mississauga, Ontario, Canada). The membrane was blocked with 2% BSA/PBS with 0.05% Tween 20 (blocking buffer) at 4°C overnight and then incubated with 1μg/ml anti-IL10 antibody (SXC5) (Mosmann et al., 1990) in blocking buffer at room temperature for 1 hr. The membrane was washed with 0.2% Tween 20 in PBS (PBST) and incubated with 1μg/ml Biotin-SP-conjugated goat anti-rat IgG in blocking buffer for 1 hr. The membrane was then washed with PBST, and incubated with 0.02% peroxidase-conjugated streptavidin in blocking buffer for 1 hr. After washing the membrane with PBST, the IL10 bands were visualized by soaking the membrane in the substrate solution, AEC in 0.1M CH_3COONa with 0.4mM H_2O_2, pH 5.0. Colour development was stopped by washing with water.

Measurement of the in vivo half life of IL-10

Naive BALB/c mice were injected ip, sc, or iv with 100μg per mouse of JE 9-2 IL-10. Tail blood was collected with a microtainer serum separator (Becton Dickinson, USA) in a 2 to 4 hr intervals. The blood was set at room temperature for half hour, and centrifuged to separate the serum from the cells. Serum were then tested for IL-10 by ELISA or Bioassay. The time course of the blood IL-10 levels was plotted as a linear curve for each individual animal, and the half-life values were calculated from the slopes of individual curve-fitted lines (Cricket Graph III, Computer Associates International, Islandia, NY). Control mice were injected with PBS, their blood samples were collected and the circulating IL-10 measured as described above.

Th1 DTH assay

This assay was performed as described previously (Cher and Mosmann, 1987). Resting Th1 cells (2 weeks after antigen stimulation) were washed in RPMI and resuspended in PBS. The Th1 cells ($5x10^5$ to 10^6 cells with appropriate Ag) were injected s.c. in a volume of 30μl into the left hind footpads of naive BALB/c mice using a 30G1/2 needle. KLH was used at 30μg/foot and CRBC at $1.5x10^7$ cells per foot. The right hind footpad was injected with control samples, consisting of an equal number of the Th1 cells in PBS without antigen, or PBS alone as a control for alloantigen-specific clones. Footpad swelling was measured and expressed as the percentage increase of the experimental foot over the control foot. In some experiments, the animals were sacrificed and their hind feet were cut off at the fur line. The percentage weight increase of the experimental foot over the control foot was recorded. Each cage contained mice from different experimental groups, in order to randomize any cage effects. In most experiments, the animals were coded and measured blind: Blind and non-blind experiments yielded similar results. A two-tailed student's t-test was performed on the data (show as % above control in this thesis and mm thickness in the publication (Li et al., 1994) and significance was assumed at $p<0.05$. Other statistic analysis was also used and indicated in the corresponding figures.

SRBC DTH assay

BALB/c mice were immunized s.c. with 10^8 SRBC/mouse, with or without CFA. Fourteen days later, mice were challenged with 2×10^6 SRBC/mouse in a volume of 30µl into the left hind footpad, with the right hind footpad receiving an equal volume of PBS. The measurement of footpad swelling and calculations were the same as those for the Th1 DTH assay.

Footpad histology

Twenty-four hr after DTH induction, mice were terminated, their feet were collected and fixed with Formaldehyde (Fisher Scientific, Pittsburgh, PA) over night. The tissue slicing and H-E staining were done in the pathology laboratory in the University of Alberta Hospital.

Footpad cytokine extraction

Twenty-four hr after Th1 cells were injected into mouse footpads, the animals were sacrificed and their footpads were cut off. The footpads were minced and incubated in HBSS at 4°C for about 1hr to allow cytokines release from the tissue. In some experiments, the footpads were digested with 1mg/ml of collagenase I and 25µg/ml of DNase at 37°C for 30 min. The supernatants were collected after centrifugation at 5,000xg for 15 minutes. The cytokine levels were tested in the supernatants by ELISA (IL2, IL6, IL10 and IFNγ) or a bioassay (TNF/LT).

Measurement of vascular permeability

0.1ml of 1% Evans Blue in PBS was injected iv 18hr after injection of Th1 cells. Two hr later the animals were sacrificed, the right ventricle cut open and perfusion carried out via the left ventricle with PBS containing 0.4% heparin (Sigma) until the control feet blanched. A modified method based on (Udaka et al., 1970) was used for Evans Blue measurement. Footpads were excised and Evans Blue was extracted by incubating the footpads in formamide (Fisher) at 37°C overnight. The Evans Blue in the footpad was quantitated by measuring the absorbance of the supernatant at 650 nm with a spectrophotometer (Molecular Devices, Menlo Park, CA, USA). Vascular permeability was represented by the amount of Evans Blue extracted.

C. Materials and Methods Specific for Chapter V

CD8 T cell purification

Splenocytes from naive mice were incubated with a rat-anti-mouse CD4 (1μg/10^6 cells) and a rat-anti-mouse NK1.1 antibody (1μg/10^6 cells) in 1 ml of RPMI with 8% FCS on ice for 20 min. These cells were then loaded onto a Rat T glass bead column (Biotex Laboratories Inc, Edmonton, Alberta) coated with sheep-anti-rat immunoglobulin (cross reactive with mouse immunoglobulin). This procedure removed most of the macrophages, B, NK, and CD4 T cells. Cells that passed through the column were 80-90% CD8 T cells as analyzed on a FACScan (Becton Dickinson, Mountain View, California). Cells collected from the column were then stained with rat-anti-mouse Ly3-PE and rat-anti-mouse CD44-FITC, and further purified by sorting CD8 positive and CD44 low (Tough and Sprent, 1994) cells on a Coulter EPICS Elite cell sorter (Coulter Electronics, Hialeah, Florida). The purity of the CD8 T cells was > 99% after this procedure.

Allo-reactive Tc1 and Tc2 cell generation

Purified naive CD8 T cells ($1x10^3$/well) were stimulated with $1x10^4$/well irradiated (10,000 RADs) M12.4.1 or J774 cells in flat-bottom 96-well tissue culture plates (Costar, Cambridge, MA, USA) in 200µl/well of RPMI with 8% FBS and 1ng/ml IL-2 in the presence of different cytokines. To generate Tc1 cells, IL-12 was used as 1:200 of the COS transfected supernatant, while the Tc2 culture contained 30ng/ml IL-4 and 50µg/ml anti-IFNγ (XMG1.2). Media (120µl) were replaced on d 3 and 5, and the cells were ready to be used by d 7. In some experiments the Tc1 and Tc2 cells were kept in IL-2 containing media for an additional 2 wk with their media changed every 3-5 days. Aliquots of Tc1 and Tc2 cells ($2x10^5$/well) were stimulated with Con A (5µg/ml) for 24hr, and the secreted cytokines were measured by ELISA to confirm the cytokine production of the T cells. Additional aliquots of Tc1 and Tc2 cells were stained with anti-CD4-FITC and anti-CD8-PE antibodies, and their surface antigens were checked by FACScan.

Tc1 and Tc2 cloning

Tc1 and Tc2 clones were generated either by limiting dilution from purified naive CD8 T splenocytes, or by single T cell culture (add a single CD8 naive T cell into each well of a 96-well plate by FACS sorting). CD8 T cells were stimulated with allogeneic cells, J774 ($H2^d$), in Tc1 or Tc2 differentiation conditions for 7 d. These cells were further restimulated every 7 d with their media changed every 3 to 5 d. The cytokine profiles of the cloned cells were checked by ELISA of their Con A stimulated supernatants, and their CD4/CD8 expression checked by FACScan analysis.

CD4 T cell purification

Splenocytes from naive mice were incubated with a rat-anti-mouse CD8 (1µg/10^6 cells) in 1 ml of RPMI with 8% FCS on ice for 20 min. These cells were then loaded onto a Rat T glass bead column coated with sheep-anti-rat immunoglobulin (cross reactive with mouse immunoglobulin). Cells collected from the column were then stained with rat-anti-mouse L3T4-PE and rat-anti-mouse CD44-FITC, and further purified by sorting CD8 positive and CD44 low (Tough and Sprent, 1994) cells on a Coulter EPICS Elite cell sorter. The purity of the CD4 T cells was > 99% after this procedure.

Allo-reactive Th1 and Th2 cell generation

Purified naive CD4 T cells ($1x10^4$/well) were stimulated with $5x10^4$/well irradiated (10,000 RADs) M12.4.1 or J774 cells in flat-bottom 96-well tissue culture plates in 200μl/well of RPMI with 8% FBS and 1ng/ml IL-2 in the presence of different cytokines. To generate Th1 cells, IL-12 was used as 1:200 of the COS transfected supernatant and anti-IL-4 (11B11) was added at 30μg/ml, while the Tc2 culture contained 30ng/ml IL-4 and 50μg/ml anti-IFNγ (XMG1.2). Media (120μl) were replaced on d 3 and 5, and the cells were ready to be used by d 10. In some experiments Th1 and Th2 cells were kept in IL-2-containing media for an additional 2 wk with their media changed every 5 d to generate resting Th cells. Aliquots of Th1 and Th2 cells ($2x10^5$/well) were stimulated with Con A (5μg/ml) for 24hr, and the secreted cytokines were measured by ELISA to confirm the cytokine production of the T cells. Additional aliquots of Tc1 and Tc2 cells were stained with anti-CD4-FITC and anti-CD8-PE antibodies, and their surface antigens were checked by FACScan.

The CD8 and CD4 DTH assay

The adoptively transferred DTH assay was performed as previously described (Cher and Mosmann, 1987). Briefly, d 10 Tc1 or Tc2 (Th1 or Th2) cells were washed twice with RPMI, and resuspended according to required concentrations. The cells were then injected sc (30μl) into the left hind footpads of naive mice bearing target MHC molecules using 28G needles (Becton Dickinson and Company, Franklin Lakes, New

Jersey). The right hind footpads of these mice were injected with 30μl of RPMI as internal controls. Footpad DTH was also induced by injecting Tc1 or Tc2 cells with their APCs into the left hind footpads of syngeneic mice, the right hind footpads of these mice were injected with Tc1 or Tc2 cells only as internal controls. Both hind feet were measured with a Mitutoyo gauge at different times post-injection. Footpad swelling was expressed as the percentage increase of the experimental foot over the control foot. In all the experiments, each cage contained mice from different experimental groups to randomize any cage effects. The measurement was done with all the animals coded in a blinded manner.

Measurement of vascular permeability

The test for vascular leakage was performed as previously described in Materials and Methods Specific for Chapter IV, except that Evans Blue was injected iv 12 hr after the DTH induction, and animals were sacrificed and their feet collected 6 hr later.

Footpad cell extraction

Mice were terminated at different time points after DTH induction, and their footpads were cut off as close to the bone as possible. Each footpad was put into 0.5 ml of RPMI with 8% FBS in a eppendorf tube. The footpads were cut into small pieces with a pair of surgical scissors (about 150 cuts/footpad). The tubes were then centrifuged at 2,000 x g for 20 min. The tissue pellets were resuspended in 0.5 ml of HBSS with 20mM HEPES and 8% FBS. The tissue pieces were digested with 1mg/ml of collagenase V (340U/ml, Sigma) at 37°C for 30 min on a roller plate. The tissues were then further dissociated by rubbing on a mesh, and washed twice with RPMI containing 8% FBS. Live cells were selected by a single-step Ficoll layer and then washed twice with RPMI. The cells were resuspended in RPMI with 1% of FBS, counted, centrifuged onto glass slides using a SHANDON Cytospin 2 centrifuge (Johns Scientific), and air dried overnight. Cells on some of the cytospin slides were stained with Eosin Y and Methylene Blue using a LeukoStat^{TM} stainkit (Fisher). Neutrophils and eosinophils in each slide were counted with all the slides coded. For immunochemical staining, cells on cytospin preparations were fixed with either 4% PFA for Gr-1 staining, or acetone (Fisher) for Mac-3 staining, the slides were then washed with PBS twice, air dried at room temperature and stored at -79°C for later use.

Immunohistochemical staining

Before staining, cytospin slides taken from -79°C were set at room temperature for 20 min, fixed once again with either 4% PFA or acetone on ice, and washed twice for 10 min each with ice cold PBS. The cells on the slides were then incubated with 50µl/cytospin area of 0.3% H_2O_2/H_2O on ice for 3 min, washed with cold PBS as before, incubated with 50µl/area of 1% H_2O_2/PBS at room temperature for 10 min, and again washed with cold PBS. The cells were then incubated for 2hr at 4°C in 25µl with the following mAbs to detect different cell surface markers: anti-Mac-3 for macrophages (Ho and Springer, 1983), 25µg/ml; and anti-Gr-1 for granulocytes (Hestdal et al., 1991), 10µg/ml. For control staining, cells were incubated with same concentration of a corresponding isotype-matched rat IgG. The slides were then washed with cold PBS and incubated with 10µg/ml, 25µl/sample of biotinylated rabbit anti-rat IgG (H+L) (Vector laboratories, Inc., Burlingham, CA) at room temperature for 30 min, and then washed with cold PBS. The slides were further incubated with 25 µl/sample of streptavidin-peroxidase conjugate (ZYMED Laboratories Inc., CA) at room temperature for 30 min, and again washed with PBS. Each sample area was then overlaid with 50 µl AEC substrate solution (ZYMED), incubated at room temperature for 20 min, and washed with distilled water for 5 min. The slides were then counter stained with 1:8 dilution of hematoxylin solution (Sigma) for 1 min, washed with tap water for 1 min, air-dried and coverslip-mounted with GVA MOUNT (ZYMED).

Systemically transferred DTH

H2^{b}-anti-H2^{d} Tc1 or Tc2 cells (10^7/mouse) were injected iv into C57BL/6J mice (H2^{b}), 1 hr later, these mice were challenged with J774 cells (H2^{d}, 10^6/mouse) in their left hind footpads with their right hind footpads receiving RPMI as controls. In the control group, mice received RPMI iv, and challenged with J774 cells in the left hind footpads 1 hr later. Footpad swelling was measured at different times post-challenge.

Tc1 and Tc2 migration assay

H2^{b}-anti-H2^{d} Tc1 or Tc2 cells (5×10^5) were injected together with an equal number of J774 (H2^{d}) cells into the left hind footpads of C57BL6 mice (H2^{b}) to initiate a local inflammatory reaction. The right hind footpads of these mice were injected with RPMI as controls. Six hr after the cell injection, these mice were injected iv with 10^7 of ^{51}Cr labeled Tc1 or Tc2 cells (H2^{b}-anti-H2^{d}). Another 6 hr later, mice were sacrificed, and both the inflammatory and the control hind feet were collected. The radioactivity in each foot was measured by gamma counting to represent the migrated Tc cells.

Footpad cytokine extraction

Mice were terminated at different time points after DTH induction, and their footpads were cut off as close to the bone as possible. Each footpad was put into 0.5 ml of RPMI with 8% FBS in a eppendorf tube. The footpads were cut into small pieces with a pair of surgical scissors (about 150 cuts/footpad) and then incubated at 37°C for 45 min to allow cytokine release from the tissue. The tubes were then centrifuged at 2,000 x g for 20 min, and the supernatants were collected for cytokine detection. If not used immediately, supernatants were stored at -79°C for later use. The potential loss of cytokines in footpad extracts was tested by adding known amounts of cytokines into the extracts, and after 1hr of incubation at 4°C the cytokines were tested from the extracts by either ELISA or bioassay. The recoveries of different cytokines were: IL-2, 136%; IL-4, 85%; IL-5, 169%; IL-10, 125%; IFNγ, 151% and TNF/LT,.217%.

Footpad cytokine detection by ELISA

Most cytokines were measured by double mAb sandwich ELISAs. To reduce the assay background, 3% normal rat serum was added to all the ELISA solutions except for the coating antibodies, and room temperature incubation was used instead of 37°C. To confirm the specificity of the signals detected by ELISA, extra control experiments with mismatched antibodies (i.e. wrong specificity but the same isotype, for example, anti-IFNγ was used as coating antibody, and biotinylated anti-IL4 (11B11, rat IgG1) was used as second antibody instead of anti-IFNγ (XMG1.2, rat IgG1).) were performed for all the cytokine ELISAs. Signals of the footpad supernatants detected by the mismatched antibodies were not higher than the assay background.

Anti-cytokine antibody effects on DTH induced by Tc1 or Tc2 cells

Anti-IL-4, anti-IFNγ or the isotype control antibody GL119 was purified using a protein G column, and was stored in PBS at -79°C. All the antibodies contained <0.5ng endotoxin/1mg, tested using a endotoxin kit (Sigma). All the antibodies were administered ip in 0.1 ml PBS at the same time as the footpad DTH induction. In another control group, mice received an equal volume of RPMI ip at the same time as DTH induction. DTH was induced as described above.

Generation of cytokine-deficient Tc1 cells

The cytokine-deficient Tc1 cells were obtained as described (Sad and Mosmann, 1995). Briefly, d 7 Tc1 cells ($2x10^6$) were cultured in 30 ml of RPMI containing 8% FBS, 1ng/ml of IL-2 and 10ng/ml of IL-4 for an additional wk. Aliquots of these cells

(2×10^5/well) were stimulated with either Con A (5μg/ml) or M12 cells (1:1 ratio) for 24 hr, and the cytokines (IL-2 and IFNγ) of these cells was measured by ELISA.

D. Materials and Methods Specific for Chapter VI

Generate Th1, Th2, Tc1 and Tc2 cells from perforin-deficient mice

The perforin-deficient Th1, Th2, Tc1 and Tc2 cells were generated the same ways as those for the control corresponding cells showed in the materials and methods specific for chapter V, except that the naive CD8 T cells were purified from perforin-deficient mouse splenocytes.

Cytotoxicity assay

Day 10 Tc1 or Tc2 cells were used as the effector cells in this assay. Target cells (M12.4.1) were radiolabelled by incubating 10^7 cells with 100μCi of ^{51}Cr in 100μl medium at 37°C for 45 min. Cells were then washed with 50ml RPMI twice, and left at 37°C for another 30 min in 30 ml medium. Various ratios of effectors to targets were co-cultured in round bottom tissue culture plates in 100μl RPMI with 8% FBS. Supernatants were collected 4 hr after the culture, and the radioactivity was detected by gamma counting. The percent cytotoxicity was calculated using the following formula: (cpm experimental-cpm spontaneous)/(cpm total-cpm spontaneous)x100

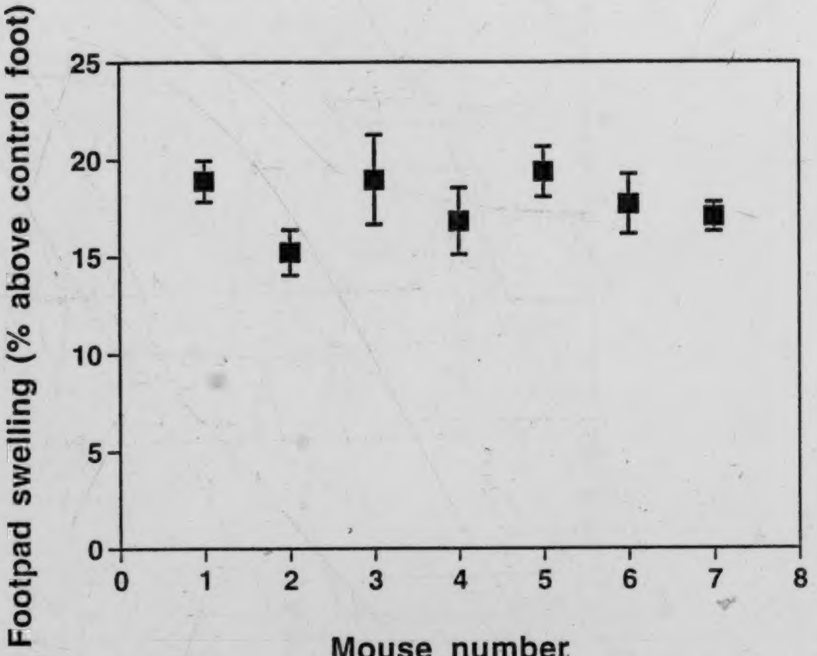


Figure 3.1 Quality control of footpad measurement. In this experiment, footpad swelling was induced by injecting Tc1 cells (10^6/mouse) into the left hind footpads of BALB/c mice. An equal volume of RPMI was injected into the right hind footpads of these mice as internal controls. Each mouse was placed into a separate clean cage before the measurement of their footpad swelling 22 hr after the T cell injection. Swelling of both hind feet of each mouse was measured and the numbers recorded in a card, the card was folded and kept in the corresponding mouse cage. The cages were then rearranged and another two rounds of measurements were performed as described above. Each point represents the mean ± SD of the three measurements of footpad swelling for each mouse.

Chapter IV

IL-10 inhibits Th1 clone- and SRBC-induced footpad DTH

(Most of the data presented in this chapter have been published in an article entitled "IL-10 inhibits cytokine production, vascular leakage and swelling during Th1-induced delayed type hypersensitivity" by Li Li, John F. Elliott and Tim R. Mosmann in *J. Immunol.* (153:3967-3978)

A. Introduction and rationale

IL-10 was originally isolated from mouse Th2 cells as cytokine synthesis inhibitory factor (CSIF) of Th1 clones (Fiorentino et al., 1989). It inhibits the cytokine production of Th1 cells stimulated by macrophages (Fiorentino et al., 1991b; Fiorentino et al., 1989) or dentritic cells (Macatonia et al., 1993; Kawamura and Furue, 1995) as APCs. The effect of IL-10 on Th1 cells is indirect (Fiorentino et al., 1991b), partially through the inhibition of IL-12 production (Germann, Rude and Mosmann, unpublished data) and B-7 expression (Ding et al., 1993; Flores Villanueva et al., 1994; Chang et al., 1995) of APCs. In addition, IL-10 also inhibits macrophage stimulated Th1 cell proliferation (de Waal Malefyt et al., 1991b; Ding and Shevach, 1992). IL-10 suppresses cytokine production (Fiorentino et al., 1991a) and microbicidal activities (Gazzinelli et al., 1992c; Oswald et al., 1992a; Cunha et al., 1992) of macrophages, and pro-inflammatory cytokine and chemokine synthesis by neutrophils (Wang et al., 1994; Kasama et al., 1994). Macrophages and neutrophils are the major antigen-nonspecific effector cells participating in the DTH reaction. Therefore, IL-10 could potentially inhibit DTH or other DTH-like inflammatory responses through its inhibitory effects on Th1 cells, macrophages and neutrophils.

In vivo studies of IL-10 support its potential anti-DTH and anti-inflammatory functions. IL-10 mRNA is often expressed in situations of increased Th2 type of responses and suppressed Th1 type of responses (Yamamura et al., 1991; Heinzel et al., 1991; Romani et al., 1993). IL-10 inhibited LPS induced septic shock, and suppressed TNF release in vivo (Howard et al., 1993). An IL-10-containing keratinocyte supernatant inhibited the initiation of a DTH reaction against alloantigens (Rivas and Ullrich, 1992). Furthermore IL-10-deficient mice developed a colitis as the major abnormality (Kuhn et al., 1993). All these results indicated that IL-10 could inhibit the Th1 response or other inflammatory responses in vivo.

IL-10 is an acid-sensitive protein whose bioactivities lost below pH 5.5. The molecular weight of mouse IL-10 is 35-40 kDa with the pI of 8.1 (Fiorentino et al., 1989). It functions as a noncovalent-linked homodimer (Moore et al., 1993). The monomer of mouse IL-10 contains 160 amino acids with molecular weights of 17, 19 or 21 kDa, due to different N-linked glycosylations (Moore et al., 1990a). Glycosylated and nonglycosylated mouse IL-10 function similarly in vitro (Mosmann, 1994b), but the potential in vivo effect of the glycosylation on the IL-10 functions is unknown. The mouse rIL-10 used for this study was produced by a BW5147 mouse lymphoma cell line transfected with mouse IL-10 cDNA. This cell line (named as JE 9-2) was kindly provided by Dr. J.F. Elliott.

One of the DTH models used in this study was the adoptively transferred DTH induced by injecting Th1 clones with their corresponding antigens into footpads of naive mice (Cher and Mosmann, 1987). This adoptively transferred DTH was antigen specific and MHC restricted. The footpad reaction was less indurated with a transient peak swelling around 24 hr after Th1 cell injection. Histologically it was dominated with polymorphonuclear cell infiltration (Fong and Mosmann, 1989). Collectively the characteristics of the Th1 cell induced DTH made it resembling a Jones-Mote DTH.

Furthermore, the footpad swelling was partially inhibited by the anti-IFNγ Ab, suggesting that IFNγ was an important regulator in this responses. The second DTH model used was induced by priming and challenging mice with sheep red blood cells (SRBCs), which is a commonly used mouse DTH system.

The objectives of the this study were to directly investigate IL-10 effects on the effector stage of DTH responses using these two mouse DTH models, and to further address the potential mechanisms of the IL-10 effects on DTH responses.

B. Results

JE 9-2 cells produces high levels of mouse rIL-10 which is biologically active and glycosylated.

JE 9-2 cells secreted high levels of IL-10 (Figure 4.1). In RPMI media with 10% of FBS and 5μM of MTX (the purpose of using MTX was to selectively grow high IL-10 producing clones, since IL-10 gene was co-transfected with dihydrofolate reductase gene), 10^6/ml JE 9-2 cells produced above 50,000 U/ml of IL-10, which was generally 50 times more than that produced by the same number of COS transfectants (700-900 U/ml).

In order to make the later purification easier, the growth condition of JE 9-2 cells was modified to minimize the contaminated protein while maintain the IL-10 production. RPMI media with different concentrations of FBS as well as the ITS/RPMI serum free medium were tested for IL-10 production. The levels of IL-10 production were fairly consistent among different conditions (Figure 4.1), and JE 9-2 cells secreted similar amounts of IL-10 in ITS serum-free as in medium with 10% FBS. Since the ITS serum free medium contains the least other proteins, it was then chosen to grow large numbers of JE 9-2 cells.

The JE 9-2 IL-10 was tested by a bioassay to confirm its biological activity. One of the functions of IL-10 is to inhibit IFNγ production by activated NK cells (Hsu et al., 1992). High concentrations of IL-2 mainly activate NK cells in a splenocyte culture, therefore the IL-2 (10ng/ml) stimulated splenocytes were used as a source of NK cells to test the inhibitory effect of IL-10. Equal concentrations (determined by ELISA) of COS (Moore et al., 1990b) and JE 9-2 IL-10 were diluted sequentially and incubated with IL-2 activated splenocytes. The two IL-10 samples showed similar inhibition of IFNγ production (Figure 4.2). Since the COS IL-10 was known to be biologically active, and the ELISA unit of the JE 9-2 IL-10 was based on the COS IL-10 standards, this result indicated that the ELISA and bioassay measurement of the JE 9-2 IL-10 agreed to each other, and the JE 9-2 IL-10 expressed a similar biological activity as COS IL-10. This result was also agreed with the finding that the Abs used in the IL-10 ELISA only detected natural but not denatured IL-10 (Mosmann et al., 1990). Therefore, in most of the later studies, IL-10 ELISA was used to measure the concentration of JE 9-2 IL-10, due to its convenience for operation.

The molecular weight of the JE 9-2 IL-10 was tested, and compared with the COS cell expressed IL-10, which is similar to the natural IL-10 (Moore et al., 1990b). Western blot was performed on IL-10 monomers separated by SDS-PAGE. JE 9-2 IL-10 consists mainly three polypeptides with the molecular weights of 17, 19 and 21kD, these components are similar or identical to the differentially glycosylated monomers of COS cell expressed mouse rIL-10 (Figure 4.3). Later the JE 9-2 IL-10 was also compared to natural IL-10 produced by a Th2 clone, and the molecule weights of JE 9-2 IL-10 monomers were also similar to those of the natural IL-10 (Figure 4.5). These results indicated that the JE 9-2 IL-10 probably had similar post-translational modifications to the natural IL-10.

Purification of mouse recombinant IL-10 produced by JE 9-2 cells

The acid liability of IL-10 makes it difficult to purify the bioactive protein through an immunoaffinity column using a simple acid elution, therefore other chromatographic methods have to be tested. A combination of an anion exchange (Mono Q 10/10, Pharmacia) and a size exclusion (Superdex 75, Pharmacia) column was first used. After these two steps of purification, most of the proteins were removed from the

IL-10 enriched fractions except a 30 kD protein that co-migrated with the peak fractions of IL-10. A hydrophobic interaction column (Phenyl Sepharose CL-4B, Pharmacia) was then tried to further purify the IL-10, but failed to separate the 30 kD contaminated protein from IL-10.

Seven dye columns (column media purchased from Sigma)were tested next, and a reactive blue 3GA column successfully removed the 30 kD protein from the IL-10 enriched fractions. The combination of blue 3GA and Mono Q 10/10 was sufficient to obtain a satisfactory IL-10 purification (Figure 4.4). The proteins in the final elusion fractions was proportionated with the IL-10 concentrations measured by ELISA. As the column capacity of the blue 3GA is larger than that of the Mono Q, the 3GA column was often used as the first step for IL-10 enrichment. Thus, IL-10 was sequentially enriched from JE 9-2 supernatant by the blue 3GA and the Mono Q 10/10 columns. The eluted fractions were tested for IL-10 by ELISA after each step of purification. The IL-10 specific activity (ELISA units/mg protein) was increased by each step and the overall recovery was 40-80% (Table 4.1).

IL-10 containing samples were also analyzed by SDS-PAGE and western blot after each step of purification. The IL-10 bands revealed by western blot in the final IL-10 preparation corresponded well with the proteins stained by Coomassie blue (Figure 4.5). As assessed by scanning of the Coomassie-stained gel, these two bands represented >95% of the total protein. The specific activity of the purified IL-10 was 3.9×10^6U/mg as tested by ELISA and BioRad protein assay, and 5.3×10^6U/ml as determined by the extinction coefficient of the protein (Howard et al., 1993). These values are similar to that reported previously for bacterial expressed rIL-10 (Howard et al., 1993).

The biological activity of the enriched IL-10 was also tested. Compared to the COS-expressed IL-10, the bioassay units determined for the JE 9-2 crude supernatant and enriched IL-10 were similar to the corresponding ELISA units The ratios of ELISA unit to Bioassay unit for crude JE 9-2 sup, samples after 3GA dye column and samples after both dye and Mono Q columns were 1, 1.3 and 0.77 respectively. These numbers indicated that the biological activity of IL-10 was preserved during the purification. Thus, the biologically active JE 9-2 IL-10 was substantially enriched by a two-step column purification.

Measurement of the in vivo half life of JE 9-2 rIL-10

In order to determine the optimal route of IL-10 administration for in vivo studies, the in vivo kinetics of IL-10 loss were tested. IL-10 (100μg/mouse) was injected into BALB/c mice either ip, sc or iv, and tail blood samples were collected at 2-4 hr intervals. The serum IL-10 levels were measured by either ELISA, or by bioassay.

With all three types of systemic administration, a high level of IL-10 was shown in tail blood within 1 h after injection (Figure 4.6), suggesting that the injected IL-10 equilibrated into the circulation rapidly. In control animals, which were injected with PBS, the serum IL-10 levels were either undetectable (data not shown) or less than 12ng/ml during the first 12 hr after the injection (Figure 4.6a). The rate of IL-10 loss in the circulation was detected by measuring different time point's serum IL-10 by ELISA in all three types of injections. The iv injected IL-10 decreased faster in circulation. The half life was about 1.6 hr, and the serum IL-10 dropped close to the control level within 12 hr (Figure 4.6a). The IL-10 given by sc and ip injections sustained longer in the blood, with an apparent half life of 5.3± 1 (Figure 4.6b) and 2.5 ± 0.2 hr (Figure 4.6c) respectively, and the serum IL-10 stayed above control level for about 20 hr. The slower rates of loss of the ip or sc injected IL-10 may be due to slower release of IL-10 into the circulation. The serum levels of ip injected IL-10 were also detected by bioassay, and the apparent half life was 1.8 ± 0.3 hr (Figure 4.6d). Overall these results indicated that IL-

10 in vivo was relatively stable compared to some other cytokines, such as IL-2 (Muhlradt and Opitz, 1982). Therefore all these types of systemic route could be used for IL-10 in vivo administration.

IL-10 inhibits M264-15 Th1 clone induced footpad swelling

M264-15 (A. TL strain) is a long term Th1 clone specific for $H2^d$ alloantigen. The footpad DTH induced by M264-15 in naive BALB/c ($H2^d$) mice is typical among DTH induced by other Th1 clones (Cher and Mosmann, 1987), therefore the M264-15 clone was chosen as a representative to test the IL-10 effects on DTH reaction induced by Th1 clones.

The dose related effect of IL-10 on M264-15 induced DTH was first examined using a single ip injection of IL-10. IL-10, ranging from 2μg to 200μg, was administered at the same time as the M264-15 cells were injected into the naive BALB/c mice to induce footpad DTH. Footpad swelling was measured 24 hr later. IL-10 induced dose-dependent suppression of the footpad swelling up to 36% of inhibition, and at least 20 μg of IL-10 was needed to show a significant inhibitory effect (Figure 4.7). At the doses of 100 and 200 μg per mouse, IL-10 gave similar levels of suppression of the footpad swelling, suggesting that the inhibitory effect was saturated above 100 μg of IL-10 by a single ip administration.

Considering that the in vivo half life of ip injected IL-10 was around 2 hr, it is reasonable that a large initial amount of IL-10 is needed in order to sustain a functional level in the circulation during the 24-hr DTH reaction. On the other hand, smaller amounts of IL-10 given by multiple injections may also achieve a sustained effective level in the blood. Therefore IL-10 was also administered by multiple ip injections to test its inhibitory effect on M264-15 induced DTH.

IL-10 was given by triple ip injections, at 0, 8, and 16 hr after DTH induction. At the same doses (10μg to 200μg) as those for the single ip injection, IL-10 given by triple injections was more efficient in DTH inhibition. Suppression was shown at all the IL-10 doses with percentages of inhibition ranging from 41% to 52% (Figure 4.8a). When smaller amounts of IL-10 (total doses from 1μg to 40μg) were tested (Figure 4.8b), the suppression of footpad swelling showed a dose related pattern, ranging from 25% to 49%, and in this case 1μg of IL-10 significantly inhibited the footpad swelling. 10μg and 40μg of IL-10 expressed a similar suppression of the footpad swelling (49%) (Figure 4.8b), this suppression was also similar to that induced by 100μg of IL-10 (52%) (Figure 4.8a), therefore, with the triple-injection protocol, 10-40μg of IL-10 induced the maximal inhibitory effect.

The inhibitory effects of IL-10 administered by single, double and triple ip injections were compared in the same experiment. At the higher dose (100μg/mouse), IL-10 given by all the frequencies of injection significantly inhibited the footpad swelling at 24 hr, however IL-10 administered by double and triple ip injections expressed a more sustained inhibitory effect on footpad swelling (Figure 4.9a). At the lower dose (10μg/mouse) IL-10 given by both double and triple injections inhibited the 24 hr footpad swelling to about 30% (Figure 4.9b). Although the single ip injected IL-10 inhibited the 18 hr footpad swelling, this effect did not last through 24 hr.

Taken together, all the above results indicated that IL-10 inhibited Th1 cell-induced DTH. This effect can be obtained by either a single injection of a large amount, or by multiple injections of smaller amount of IL-10. However even at the optimal doses, IL-10 only gave a partial inhibition of the footpad swelling.

To test the most effective time of IL-10 administration for DTH inhibition, IL-10 (100μg/mouse) was administered by a single ip injection at different times either before or after DTH induction. In one experiment, IL-10 was administered 5 hr before, at the same time or 5 hr after the DTH induction, and only the IL-10 given at 5 hr after the DTH significantly inhibited the 24 hr footpad swelling, and this inhibition was sustained for about two days (Figure 4.10a). In another experiment, IL-10 was given at 0, 4, 8, or 12 hr after Th1 cell injection (Figure 4.10b). IL-10 administered at all the time points except for 12 hr after DTH induction inhibited the 24 hr footpad swelling, and the inhibitory effect was also sustained for about two days. This result indicated that for an effective inhibition of footpad DTH, IL-10 is needed at the early stage of DTH induction (0 to 8 hr). Although IL-10 given 12 hr after DTH induction did not inhibit the 24 hr footpad swelling, it significantly suppressed the 36 and 48 hr footpad swelling, this result demonstrated both the ongoing DTH progress and the ongoing IL-10 suppressive effect on DTH after 24 hr.

To investigate whether the effect of IL-10 varied with the number of Th1 cells injected or with the magnitude of the DTH response, different numbers of M264-15 cells were used to induce footpad DTH in BALB/c mice. The magnitude of the 24 hr footpad swelling was Th1 cell number-dependent as reported before (Cher and Mosmann, 1987). IL-10 (100μg/mouse), administered by a single ip injection, suppressed the footpad swelling induced by Th1 cells at all cell numbers tested (Figure 4.11).

Since IL-10 injected iv, ip, or sc, yielded similar serum levels of IL-10 and expressed close (within three folds) kinetics of loss in the serum, we hypothesized that IL-10 that given by any of these three routes should show similar inhibition to the footpad DTH. Therefore different routes of IL-10 administration were tested for their inhibition of footpad DTH induced by M264-15 cells. In these studies, both the 24 hr footpad swelling and the foot weight were recorded to show the IL-10 effect. IL-10 significantly inhibited the footpad DTH (measured as weight or thickness) when administered by any of these three routes (Figure 4.12).

IL-10 inhibits DTH induced by other Th1 clones

To investigate whether the inhibition of M264-15 induced DTH by IL-10 is a general effect on DTH induced by other Th1 clones, three more Th1 clones were tested. MD13-5.1 and MD13-10 are specific for CRBCs, while HDK-1 is specific for a soluble Ag, KLH (Cher and Mosmann, 1987). These Th cells were injected together with their appropriate Ags into the footpads of naive BALB/c mice. IL-10 was given either by a single ip injection of 80μg per mouse (Figure 4.13) or by triple ip injections of a total amount of 20μg per mouse (Figure 4.14). IL-10 partially but significantly inhibited the 24 hr footpad swelling in all cases, except for DTH induced by HDK-1 cells in one experiment. However the degree of inhibition of the footpad DTH induced by different Th1 clones varies.

The time course of the IL-10 effect was studied by measuring footpad swelling every 8 to 12 hr over several days. As previously reported, the peak reaction of the Th1 DTH reached at about 24 hr and the swelling usually lasted for 2 days or more (Cher and Mosmann, 1987; Fong and Mosmann, 1989). In the DTH reactions induced by different Th1 clones, IL-10 did not appear to suppress the footpad swelling for the first few hours. The strongest inhibitory effect was observed at the peak reaction, and for most of the Th1 clones, the inhibition lasted for days (Figure 4.14). Increasing amounts of IL-10 induced more inhibition of M264-15-induced DTH, but the time of maximum suppression was always at the time of peak swelling (Figure 4.14a).

IL-10 inhibits vascular permeability during the Th1 DTH response

Tissue edema is often part of the pathological changes of DTH responses. IL-10 effects on edema were tested using Evans Blue dye. The dye was injected iv 18 hr after M264-15 cells were injected to induce DTH. Two hr later the mice were terminated, perfused with heparin-containing PBS, and the Evans Blue was extracted from each footpad to measure the vascular leakage. The DTH footpads from IL-10 treated mice contained significantly less Evans Blue than footpads from control mice (Figure 4.15), suggesting that IL-10 inhibits vascular permeability during the DTH reaction. The inhibition of Evans Blue extravasation by IL-10 was 30-50%, which was similar to the inhibition of the footpad swelling or the foot weight increase. This result suggested that the major inhibitory effect of IL-10 on footpad swelling may due to the inhibition of edema.

IL-10 does not alter the cellular infiltration in 24 hr DTH footpads

Cellular infiltration is another characteristic of DTH responses. The IL-10 effects on cellular infiltration during DTH reaction were studied on the 24 hr footpad sections stained with hematoxylin and eosin (H-E). A heavy infiltrate dominated by polymorphonuclear cells was seen within or around the T cell-injected area of each DTH foot section (e.g. Figure 4.16). The size of the infiltrated area and the density of infiltrate varied among sections of each foot. The numbers of the mononuclear cells and the polymorphonuclear cells were counted in five 12.5 x 40 microscope area for each tissue section. The polymorphonuclear to mononuclear cell ratios varied between 20:1 to 2:1 in different regions of the section, possibly related to proximity to the site in which Th1 cells were injected. When the combined data from several sections of each foot were considered, no significant difference, either qualitative or quantitative, was found between the IL-10-treated and the control feet. This result suggested that IL-10 may not affect cellular infiltration in the DTH response induced by Th1 clones. Figure 4.16 showed the representative sections of 24 hr DTH feet collected from either IL-10- or PBS-treated mice.

IL-10 reduces the level of Th1 and inflammatory cytokines in the DTH footpads

In vitro, IL-10 inhibits cytokine production by Th1 cells as well as by macrophages. DTH induced by Th1 cells is partially regulated by IFNγ. Furthermore, cytokines produced by macrophages and other accessory cells, such as IL-6 and TNF, are also directly involved in inflammatory responses. Therefore the inhibition of DTH by IL-10 could be due to the inhibition of the synthesis of these cytokines. To test this hypothesis, the in vivo levels of cytokines were measured from footpad extracts. The cytokines tested included those of Th1 cells (IL-2, IL-3, GM-CSF, IFNγ and LT), macrophages (IL-6, IL-10 and TNF), as well as Th2 cells (IL-4, IL-5, IL-6 and IL-10). Among all these cytokines, the levels of IL-2, IL-6, IL-10, IFNγ and TNF/LT were elevated in the DTH feet injected with Th1 cells (Figure 4.17, Table 4.2), suggesting that these cytokines could be either correlated with Th1 cell activation or directly involved in the footpad inflammatory response. IL-10 treatment reduced the levels of IL-6, IFNγ and TNF/LT consistently, and IL-2 sometimes in the DTH footpads (Figure 4.17, 4.18 and Table 4.2). Similar suppression of the in vivo cytokines by IL-10 was shown by different routs of administration (Figure 4.17 and Table 4.2). IL-10 levels in DTH footpads were similar or higher in the control mice than the IL-10 treated mice, indicating that IL-10 may also inhibit its own in vivo levels. This result was consistent with the in vitro findings that IL-10 inhibits its own production from macrophages. (In Table 4.2, tissue extracts in Exp I and II were prepared by collagenase I digestion after mechanical disassociation. It was found that the collagenase digested most of the cytokines except for IFNγ in the footpad extracts, therefore levels of some of the cytokines in these experiments were low or undetectable).

The time course of the in vivo cytokine production during Th1 DTH response was studied at 8, 16 and 24 hr after T cell injection. Significant amounts of IL-2 (data not shown), IL-6 and TNF/LT were detected at 8 hr in the DTH footpads, while elevated IFNγ started to appear later. IL-2 (data not shown), IL-6 and IFNγ levels increased along the 24 hr post-DTH induction, while TNF/LT level peaked at 16 hr and dropped by 24 hr (Figure 4.18). In this experiment, IL-10 was administered by triple ip injections at 0, 8 and 16 hr after DTH induction. Again IL-10 inhibited both the Th1 (IFNγ and LT/TNF) and the macrophage (IL-6 and TNF/LT) cytokine levels in the DTH footpad. IL-10 blocked the accumulative increase of these cytokines in the footpads, correlated with its inhibition of footpad swelling (Figure 4.18).

Both IL-10 and anti-IFNγ antibody partially inhibit the Th1 clone-induced footpad DTH. To further test if the inhibitory effects of IL-10 was through the inhibition of the in vivo levels of IFNγ, IL-10 and anti-IFNγ was administered together. Both IL-10 and anti-IFNγ antibody, XMG 1.2, alone showed a moderate suppression of M264-15-induced footpad swelling (Figure 4.19). When IL-10 and anti-IFNγ antibody were administered together, the degree of inhibition was neither significantly greater than either reagent alone, nor greater than IL-10 plus isotype control Ab, suggesting that there was no synergy effect between IL-10 and IFNγ Ab in DTH inhibition. Therefore the inhibitory effect of IL-10 on Th1 clone-induced DTH was likely to be through the inhibition of IFNγ production.

IL-10 inhibits SRBC induced footpad DTH.

In addition to the DTH induced by Th1 clones, the effect of IL-10 was also studied on DTH induced by priming and challenging mice with sheep red blood cells (SRBCs). BALB/c mice were immunized sc with SRBCs in the presence or absence of complete Freund's adjuvants (CFA). Footpad DTH was induced by challenging these mice with SRBCs in their left hind footpads 14 d after the immunization. A single dose of IL-10 (100μg/mouse) was injected ip at the time of the antigen challenge. IL-10 inhibited the 24 hr footpad swelling induced by SRBC up to 40% (Figure 4.20). However when a smaller amount of IL-10 (20μg/mouse) was administered by triple ip injections, IL-10 failed to inhibit the 24 hr footpad swelling induced by SRBCs (Figure 4.21). This result suggested that the amount of IL-10 required and the most effective time of IL-10 administration in DTH inhibition may depend on the way how a particular DTH is induced.

The IL-10 effects on cellular infiltrate were also assessed in SRBC-induced DTH by H-E staining. Similar infiltrates were seen in sections of DTH feet from IL-10-treated and PBS-injected mice (Figure 4.22). The ratios of polymorphonuclear to mononuclear cells in the infiltrated area were 18.3 ± 8.8 for PBS control feet, and 19.0 ± 10.7 for IL-10 treated feet. This result was a collection of three counting fields for each section and six sections for each foot, of two PBS-injected and three IL-10-injected feet. Collectively these results suggested that IL-10 did not significantly inhibit the cellular infiltration in a SRBC-induced DTH response.

The in vivo cytokine profile was tested from the tissue extracts of the 24 hr DTH feet. Although IL-2, IL-6 and IFNγ levels were high in the DTH footpad injected with Th1 cells, they were not detected in the SRBC-challenged DTH footpads. TNF/LT was detected in the DTH feet, suggesting the involvement of this cytokine in the SRBC-DTH reaction. IL-10 treatment inhibited the in vivo levels of TNF/LT in the DTH footpads, but the inhibition was only significant in the mice primed with SRBC in CFA (Figure 4.23). Detection of cytokines in the control footpads was not done.

C. Summary of results

1) Mouse rIL-10 was enriched to > 95% purity by sequentially passing through 3GA blue dye and Mono Q 10/10 chromatography.

2) The in vivo apparent half life of systematically administered IL-10 was about 2 to 5 hr depending on the administration routes, and most of the circulating IL-10 was biologically active.

3) Systemically administered IL-10 inhibits Th1 clone-induced footpad swelling and tissue edema without significantly altering the cellular infiltration during the peak DTH reaction (24 hr).

4) Effective inhibition can be obtained by either a single injection of a large amount of IL-10 (e.g. 100μg/mouse) or by multiple injections of smaller IL-10 doses (e.g. 20μg/mouse total). The most effective time for single dose IL-10 administration is between 0 to 8 hr after DTH induction.

5) IL-10 significantly inhibits the levels of Th1(e.g. IL-2, IFNγ and LT) and macrophage (IL-6 and TNFα) cytokines in the DTH footpads. The endogenous IL-10 level is also elevated during a DTH response, and IL-10 may also inhibit its own production in vivo.

6) IL-10 inhibits footpad DTH induced by priming and challenging mice with SRBCs, without altering the cellular infiltration. IL-10 also inhibited the in vivo levels of TNF/LT in the DTH footpads of the mice immunized with SRBCs in CFA.

D. Discussion

The recombinant IL-10 used in this study was purified from a mammalian expression system in order to obtain material that was similar to natural IL-10 in post-translational modifications, particularly carbohydrate addition, as we considered that glycosylation of IL-10 may be important for *in vivo* function and stability. Natural IL-10 is a homodimer of polypeptides with variable glycosylation (Moore et al., 1990b). Our mammalian recombinant IL-10 consisted of three major polypeptides with mobility in SDS-PAGE very similar to natural IL-10 monomers (Figure 4.5), suggesting that it may contain similar post-translation modifications to natural IL-10. However, a recent study using *E. coli*-expressed mouse recombinant IL-10, which lacks glycosylation, inhibited the effector stage of DTH against *Leishmania major* with a similar dose range (Powrie et al., 1993). Together with the lack of glycosylation of human IL-10 (Vieira et al., 1991), these results suggest that glycosylation may not be critical for *in vivo* functions of mouse IL-10.

IL-10 has a relatively slow rate of loss in the circulation. A recent clinical trial of human IL-10 also showed a similar in vivo apparent half life as the results in this study (Huhn et al., 1996). The in vivo half lives of IL-10 administered ip or sc were significantly longer than that injected iv, suggesting that the peritoneal cavity and the subcutaneous space could serve as a slow release compartment for IL-10. For ip injected IL-10, the half lives determined by ELISA and by bioassay were significantly different: The half life measured by ELISA is longer (2.5 vs. 1.8 hours) and the apparent IL-10 amount was higher at any time point. This result suggests that a certain amount of IL-10 in the serum lost its bioactivity but was still detectable by ELISA. Since the antibodies for the IL-10 ELISA recognize the natural form of IL-10 but not some denatured forms (Mosmann et al., 1990), it is also possible that IL-10 formed complexes with other protein(s), such as soluble IL-10 receptors, which could block the biological activity but were still immunoreactive.

During immune responses, the production of IL-10 often correlates with a strong Th2 response and a inhibited cell-mediated response (Mosmann and Coffman, 1989; Mosmann, 1994a). In this study, mouse IL-10 directly inhibited the DTH induced by Th1 clones. This DTH model represents only the effector stage of DTH without requiring endogenous T cell recruitment. Activation of the injected Th1 cells by antigen and endogenous APC leads to Th1 cytokine synthesis, recruitment of other effector cells, and increase of vascular permeability leading to edema. This reaction peaks at about 24 hr and can last for days. The properties of the Th1 clone transferred DTH are quite similar to those of the Jones-Mote type of DTH. IL-10 inhibited footpad swelling, vascular leakage, and the cytokine production by T cells and other cells, but cellular infiltration was not detectably altered.

The studies of the frequencies and the time effect of IL-10 administration on DTH inhibition suggested that in order to show an effective inhibition, IL-10 is required through the development of the DTH reaction, especially at the early stage (0-8 hr) after T cell injection. In this study, relatively large amounts of IL-10 were needed for the suppression of the footpad swelling. Similar findings were reported on the IL-10 effects on the effector stage of the DTH induced by *L. major* (Powrie et al., 1993). However when administered at the initiation stage of the DTH responses, a relatively small amount of IL-10 (at ng level) gave an effective inhibition of allospecific footpad DTH (Rivas and Ullrich, 1994). These results suggested that it could be more difficult to suppress the DTH at the effector stage, when the animal has already acquired the ability to mount the reaction. Furthermore, the effective ways of IL-10 administration was different between Th1- and SRBC-induced DTH. For SRBC-DTH, a large amount of IL-10 given at the time of challenge effectively inhibited DTH, while smaller doses administered through triple ip injections, which were efficient in inhibiting Th1-DTH, failed to inhibit SRBC-DTH. Since the effector stage of SRBC-induced DTH involved local recruitment of DTH effector T cells, the cellular events in SRBC-DTH process was different from that of the Th1 adoptively transferred DTH, which may explain the difference in the IL-10 dose required and the effective time point of action between these two types of DTH responses.

IL-10 did not detectably affect either the quantity or the type of infiltrated cells in the DTH footpads, as assessed by the relatively crude method of simple H-E staining. Thus, suggests that IL-10 may not effect the gross changes in adhesion molecules and chemotaxis in the DTH models we studied. However IL-10 may express different effects on cellular infiltration depending on the experimental systems. IL-10 inhibited ICAM-1 expression on human monocytes (Willems et al., 1994), it inhibited both ICAM-1 and VCAM-1 upregulation on IL-1 activated human endothelial cells (Krakauer, 1995) and ICAM-1 expression on rat lung vascular endothelial cells (Mulligan et al., 1993). The suppression of adhesion molecule expression suggests its potential inhibitory effect on cellular infiltration. IL-10 inhibited migration inhibitory factor (MIF) production from T cells and MIF mediated macrophage activation (Wu et al., 1993), suggesting potential inhibitory effects on local macrophage infiltration. IL-10, together with IL-4, reduced the mononuclear cell infiltration during a DTH response induced by *L. major* Ag (Powrie et al., 1993). But IL-10 did not inhibit human monocyte chemotaxis induced by monocyte chemotactic and activating factor (MCAF) (Yano et al., 1995). Furthermore, IL-10 reduced neutrophil migration to the inflamed lung in rats (Mulligan et al., 1993), inhibited the adhesion of leukocytes to IL-1 activated human endothelial cells, and suppressed both neutrophil and eosinophil infiltration in a mouse airway inflammation (Zuany-Amorim et al., 1995). Collectively these results indicate that IL-10 can potentially suppress cellular infiltration in certain inflammatory responses.

DTH is often accompanied by an increase in vascular permeability, which may be mediated by a number of factors, including histamine, serotonin, prostaglandin E2 (PGE2), platelet-activating factor (PAF), substance P, IL-8 and TNF (Pober and Cotran, 1990). IL-10 may reduce vascular leakage by directly affecting the vascular endothelial cells, or by suppressing the release of one or more of these vasoactive mediators. IL-10 is known to inhibit the production of TNF and IL-8 (Fiorentino et al., 1991a; de Waal Malefyt et al., 1991a), and here I showed that IL-10 inhibited the levels of TNF/LT in the DTH footpad, thus providing a potential explanation for the ability of IL-10 to inhibit vascular leakage.

The elevation of both Th1 (e.g. IL-2, IFNγ and TNF) and non-Th1 (e.g. IL-6, IL-10 and TNF) cytokines in the DTH footpads indicates the activation of Th1 cells in vivo and the participation and activation of other accessory cells during the Th1 DTH responses. Most of the cytokines (e.g. IL-2, IL-6 and IFNγ) showed an accumulating pattern in the footpads during the 24 hr DTH reaction. The accumulation of the Th1 cytokine suggested the gradual activation of these cells in vivo or the accumulative production of their cytokines, while the increase of the non-Th1 cytokine indicated the increasing activation of endothelial cells or increasing infiltration and activation of other accessory cells, such as neutrophils and macrophages. Thus for the first 24 hr after T cell injection, cells in the footpads are continuously being activated, and their functions being upregulated. This phenomena may explain why IL-10 is consistently required during this period of time for an effective inhibition of the DTH response.

IL-10 inhibits the synthesis of inflammatory cytokines (IFNγ, IL-1, IL-6, IL-8, LT and TNFα) by Th1 cells, monocytes and macrophages in vitro (Fiorentino et al., 1989; Fiorentino et al., 1991a). During DTH induced by Th1 clones, the inflammatory cytokines were elevated in the footpad, probably because of local synthesis, and systemic IL-10 substantially reduced the levels of both the primary Th1 cytokines (e.g. IFNγ, TNF/LT) and secondary cytokines (e.g. IL-6, IL-10 and TNFα) in the footpads of ongoing DTH reactions. The TNF bioassay potentially detects TNFα, produced by Th1 cells and macrophages, and TNFβ (LT) produced by Th1 cells. Thus, the TNF measurements in footpad extracts do not distinguish between direct and indirect production of TNF, and therefore could indicate a combination of Th1 and macrophage activation. IL-6 can be produced by both macrophages and endothelial cells but not Th1 cells. The suppressive effect of IL-10 on the synthesis of these cytokines indicated its inhibition of Th1 cells and macrophages in vivo. The inhibition of IL-6 levels by IL-10 suggests that endothelial cells may not be the major source of IL-6 in the footpad, as IL-10 does not inhibit the synthesis of IL-6 by endothelial cells (Sironi et al., 1993). The combined inhibition of direct and indirect cytokine production provides a possible explanation for the ability of IL-10 to inhibit the DTH-swelling reaction. These results e.g. reduction of IFNγ levels, also suggest that the cells presenting Ag to Th1 cells in the foot are IL-10 sensitive APCs, such as macrophages (Fiorentino et al., 1991b) or dendritic cells (Macatonia et al., 1993).

Interestingly, increased expression of IL-10 was also detected in footpads undergoing Th1 DTH. This IL-10 was probably secreted by secondary cells, e.g. macrophages or keratinocytes, as mouse Th1 cells do not normally secrete IL-10. The increased expression of IL-10 was also reported in other inflammatory responses, such as endotoxemia (Frankenberger et al., 1995; Standiford et al., 1995) and inflammatory bowel disease (IBD) (Kucharzik et al., 1995). Furthermore, stronger CH and more severe tissue damage occurred in IL-10 deficient mice compared to wild type controls (Berg et al., 1995). These findings raise the possibility that the endogenous IL-10 could be responsible for quenching of inflammatory responses. The secondary expression of IL-10 later in the DTH reaction may be a cause of the rapid decline in swelling that often occurs after 24 hr. A similar role for IL-10 has been demonstrated in contact hypersensitivity (Ferguson et al., 1994).

IL-10 also inhibits the DTH induced by priming and challenging with SRBCs, which is another commonly used mouse DTH model. This result suggests that the inhibition of DTH by IL-10 could be a general effect. However the triple ip injections of smaller amounts of IL-10 that was effective in inhibiting Th1 DTH failed to inhibit this type of DTH. These results suggested that the effective amounts and time of IL-10 administration may be different among different reactions. Unlike the Th1 cell adoptively transferred DTH, which initiated by the direct activation of injected Th1 cells in situ, the effector stage of the SRBC DTH is initiated by the activation of endothelial cells and the recruitment of DTH effector T cells. Therefore the DTH processes of these two type of responses were different. No significant levels of IL-2 and IFNγ were detected, indicating that the Th1 cell frequency in the footpad may be low. However, significant levels of TNF/LT were detected in the DTH footpads, suggesting the involvement of this cytokine in the SRBC-induced DTH responses. The cellular sources of TNF/LT could be Th1 cells and macrophages. Suppression of TNF levels by IL-10 was detected in the DTH footpads of the mice immunized with CFA, suggesting that IL-10 may also inhibit the functions of either macrophages or Th1 cells this DTH model. CFA augmented the reaction, thus providing more room for studying the inhibitory effect of IL-10.

Studies of IL-10 effects on DTH or other inflammatory responses have been reported by other groups recently. Classical tuberculin-type DTH induced by *Leishmania* antigens was partially inhibited by *E. coli*-derived recombinant IL-10 (Powrie et al., 1993). IL-10 inhibits both the induction and the effector phase of DTH induced by trinitrophenyl-coupled spleen cells (Schwarz et al., 1994), and the initiation stage (Kondo et al., 1994) or the effector stage (Schwarz et al., 1994; Ferguson et al., 1994) of contact hypersensitivity (CH). Beside the DTH responses, IL-10 also suppresses other inflammatory reactions. IL-10 protects lung injury induced by immune complexes (Mulligan et al., 1993), and prevents experimental allergic encephalomyelitis (EAE) in rats, which is an autoimmune disease mediated mainly by Th1 cells (Rott et al., 1994). IL-10 suppresses inflammatory cytokine production during IBD (Schreiber et al., 1995), inflammatory arthritis (Hart et al., 1995), endotoxemia (Standiford et al., 1995) and graft versus host (GVH) diseases (Smith et al., 1995). The in vivo inhibition of inflammatory cytokines by IL-10 was also confirmed in human by an IL-10 trial (Chernoff et al., 1995). All these results together with the studies presented in this chapter demonstrate the negative role of IL-10 played in DTH and other inflammatory responses.

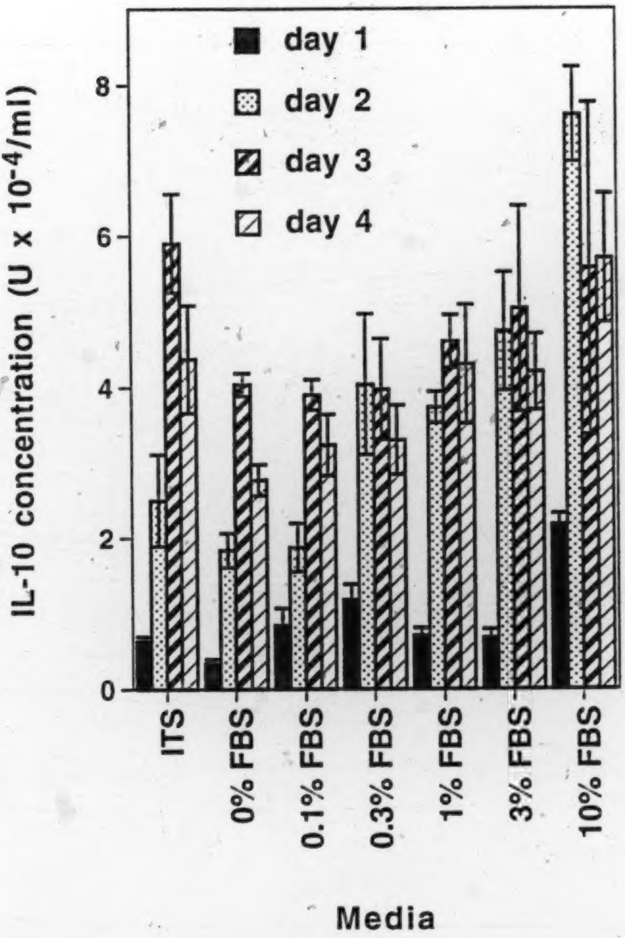


Figure 4.1 JE 9-2 cells produce high levels of IL-10. JE 9-2 cells (10^6/ml) were cultured in medium conditions indicated. Supernatants were harvested different days after the culture, and the IL-10 concentrations were tested by ELISA. Each bar represents the Mean ± SD of the IL-10 levels in three separate wells.

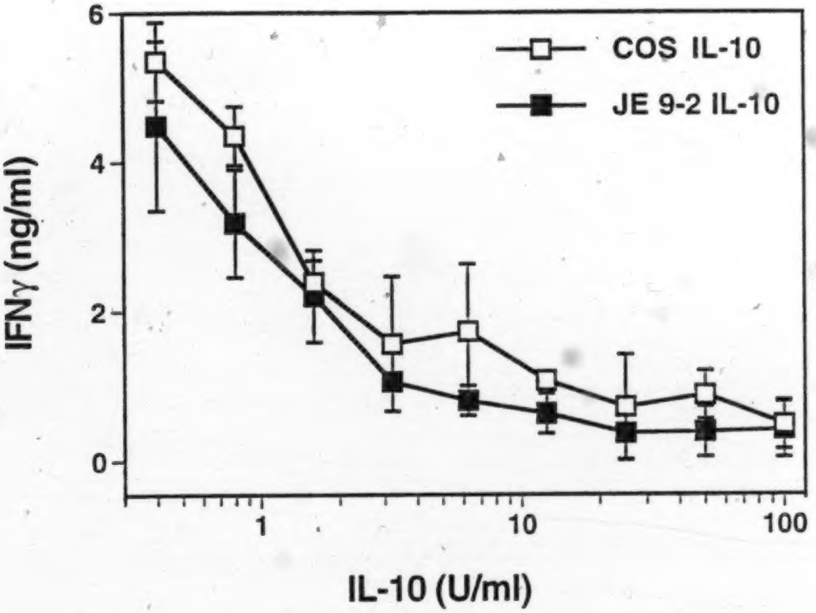


Figure 4.2. JE 9-2 IL-10 is biologically active. 100 U/ml (determined by ELISA) of JE 9-2 and COS cell expressed IL-10 were titrated two folds sequentially, and cultured with IL-2 (10 ng/ml) stimulated BALB/c splenocytes (10^6/well). Thirty-six hr later, the supernatants were collected and IFNγ concentrations were tested by ELISA. The bioactivity of IL-10 was expressed as the inhibition of IFNγ production in the supernatants. Each point represents the mean ± SD of triplicated wells.

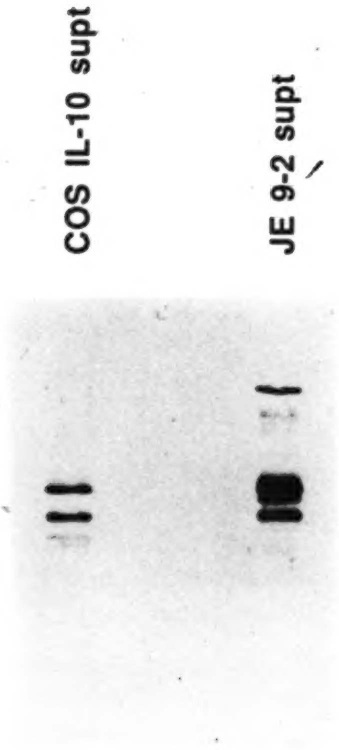


Figure 4.3 JE 9-2 IL-10 monomers have similar molecular weights to those of the COS cell expressed mouse rIL-10. Concentrated supernatants of JE 9-2 or IL-10 transfected COS cells were analyzed on a SDS-PAGE gel. Proteins on the gel were transferred onto a nitrocellulose membrane and incubated sequentially with a rat- anti-mouse IL-10 antibody (Sxc-5), a biotin-goat-anti-rat Ig antibody, and peroxidase-linked streptavidin. The IL-10 bands were visualized by soaking the membrane in an AEC substrate buffer.

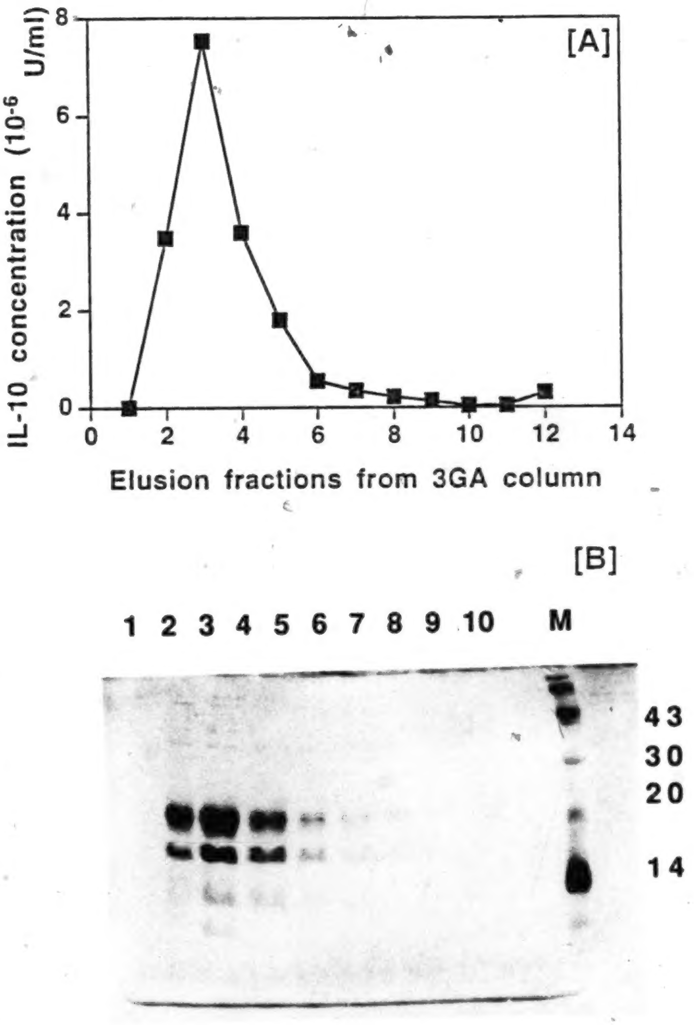
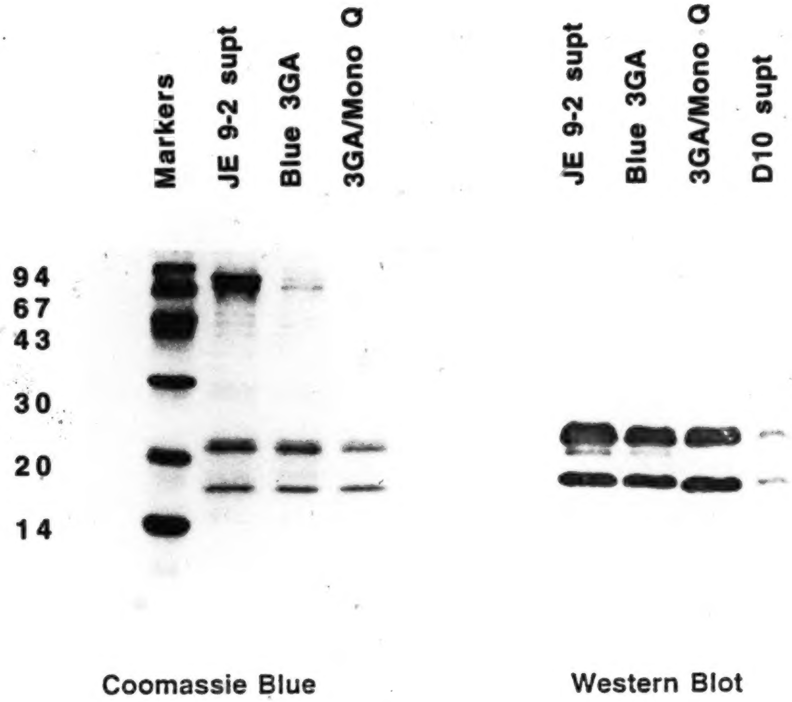


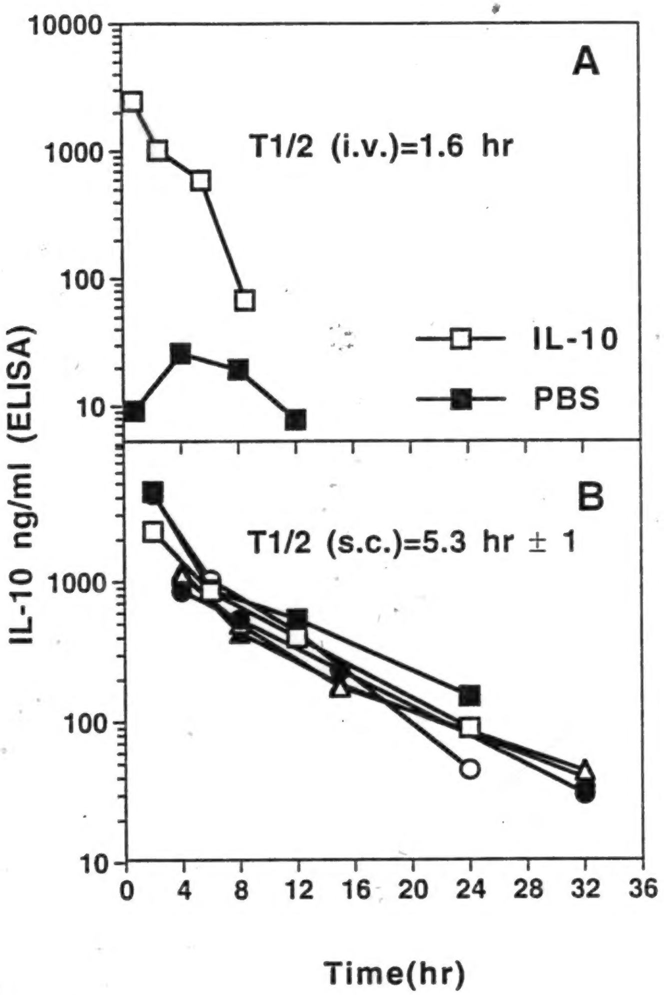
Figure 4.4 IL-10 purification by Mono Q and 3GA blue dye columns. Concentrated JE 9-2 supernatant was passed through a Mono Q column, and the IL-10 positive fractions were pooled, concentrated and loaded onto a 3GA blue dye column. The elusion fractions from 3GA blue dye column were tested for IL-10 (A), and analysed by SDS-PAGE (B). Proteins on the SDS-PAGE were stained with Coomassie blue.



Coomassie Blue

Western Blot

Figure 4.5 The purity of IL-10 after each step of purification. Aliquots of IL-10 (2000U) from each purification step were analyzed by SDS-PAGE. Gels were either stained with Coomassie blue or processed for Western blot. D10 supernatant (10µl concentrate, containing about 200U of IL-10) was used as a natural IL-10 control in the Western blot.


A
T1/2 (i.v.)=1.6 hr
IL-10
PBS
B
T1/2 (s.c.)=5.3 hr ± 1
IL-10 ng/ml (ELISA)
10000
1000
100
10
0
4
8
12
16
20
24
28
32
36
Time(hr)

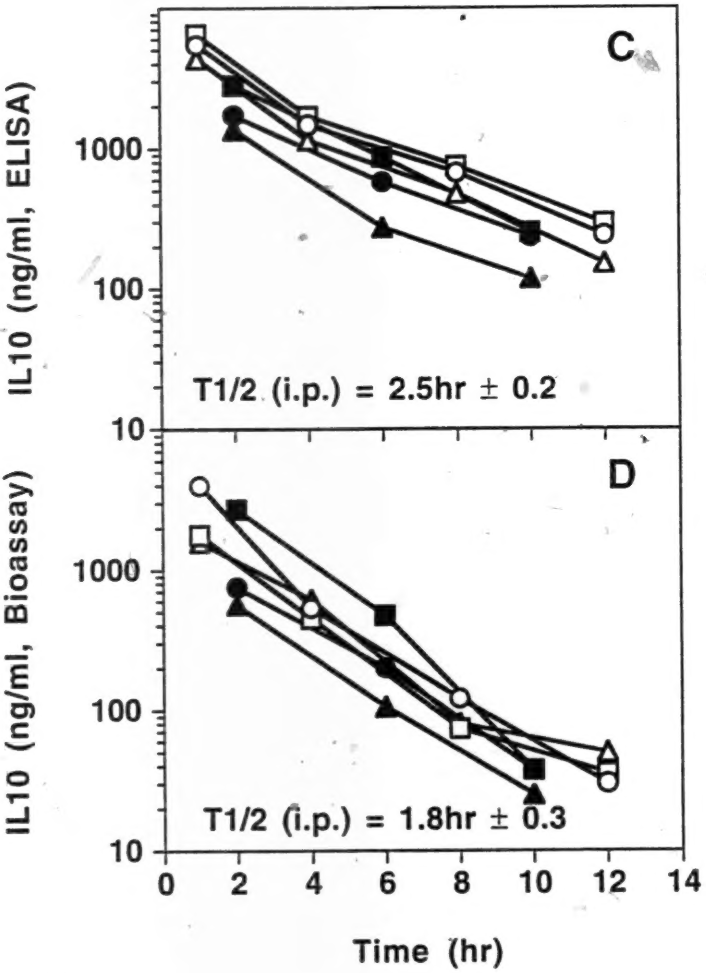


Figure 4.6. Kinetics of in vivo loss of IL-10. IL-10 (100μg/mouse) was injected into BALB/c mice i.v. (A), s.c. (B) or i.p. (C and D). A control moues received an equal volume of PBS iv (A). Tail blood was collected at 2-4 hr intervals. Serum IL-10 was measured by ELISA (A, B, and C) or bioassay (D). Each line represents an individual mouse.

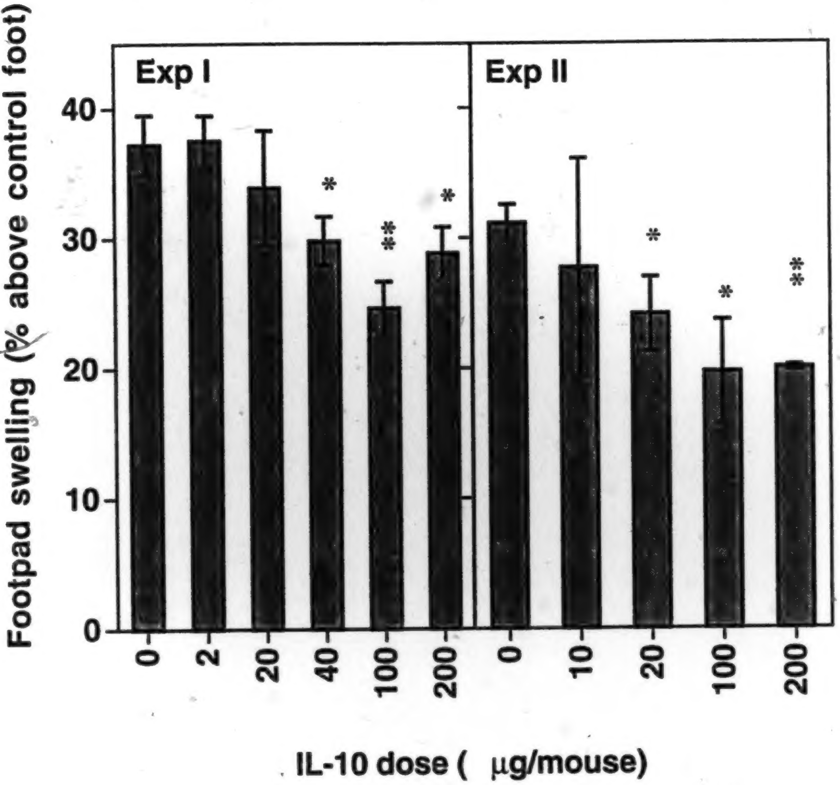


Figure 4.7. The dose effect of single ip injected IL-10 on DTH induced by M264-15 cells. DTH was induced by injecting 10^6 M264-15 Th1 cells into the footpads of naive BALB/c mice. Different amounts of IL-10 were administered by a single ip injection at the same time as DTH induction. control mice were injected ip with an equal volume of PBS. Footpad swelling were measured at 24 hr after Th1 cell injection. Each bar represents the mean ± SD, (n=3). * $p< 0.05$, ** $p< 0.005$, compared to PBS injected group. Similar significance was showed by one-way ANOVA analysis.

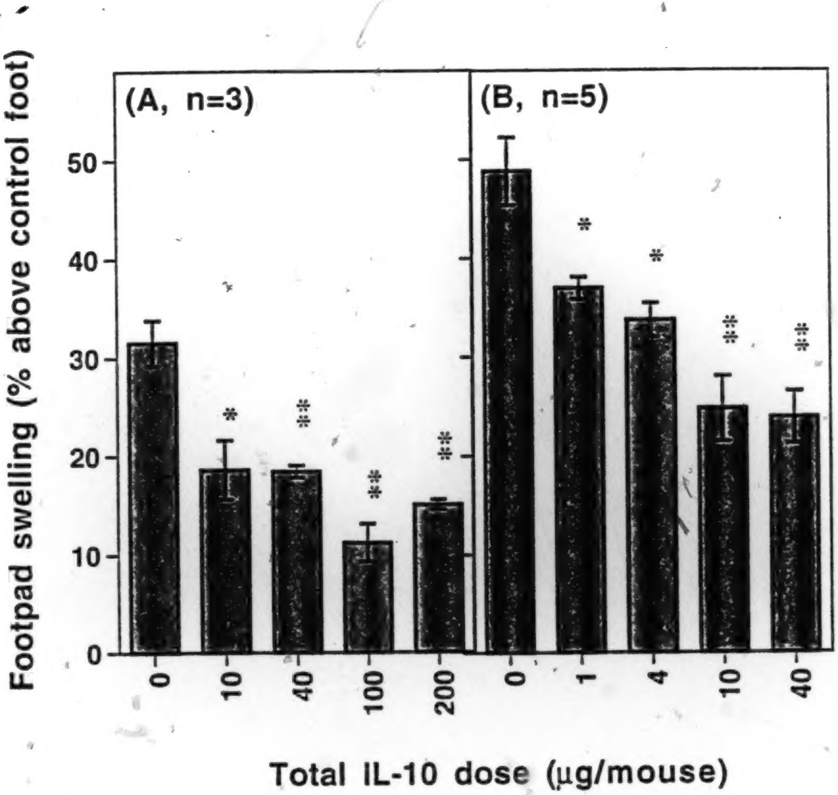


Figure 4.8. The dose effect of triple ip injected IL-10 on DTH induced by M264-15 cells. DTH was induced by injecting 10^6 M264-15 Th1 cells into the footpads of naive BALB/c mice. Different amounts of IL-10 were administered by triple ip injections at 0, 8, and 16 hr after DTH induction. control mice were injected with equal volumes of PBS. Footpad swelling were measured at 24 hr after Th1 cell injection. Each bar represents the mean ± SD. * $p< 0.05$, ** $p< 0.005$, compared to the control group.

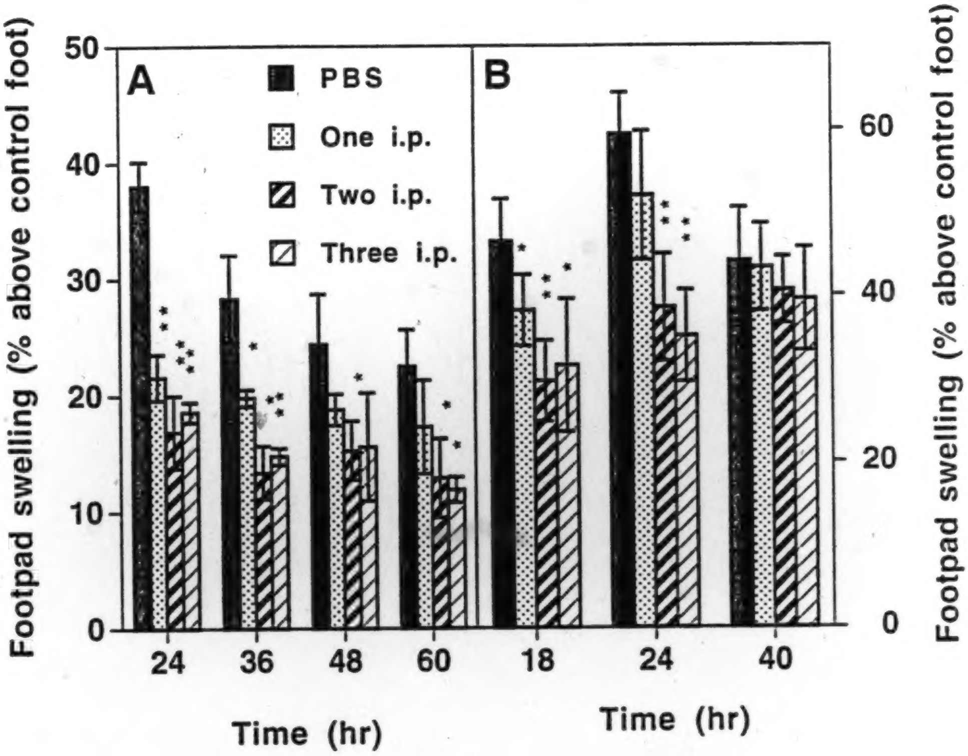


Figure 4.9. The frequency effect of IL-10 administration on DTH induced by M264-15 cells. DTH was induced by injecting 10^6 M264-15 cells into the left hind footpads of naive BALB/c mice. IL-10 was administered at a total dose of either 100µg/mouse (A, n=3) or 10µg/mouse (B, n=5) by one(at 0hr), two (0 and 12 hr) or three (0, 8, and 16hr) ip injections. Control mice were injected ip with equal volumes of PBS. Footpad swelling was measured at different times post-DTH induction. Each bar represents the mean ± SD. * $p< 0.05$, ** $p< 0.005$, compared to the control group.

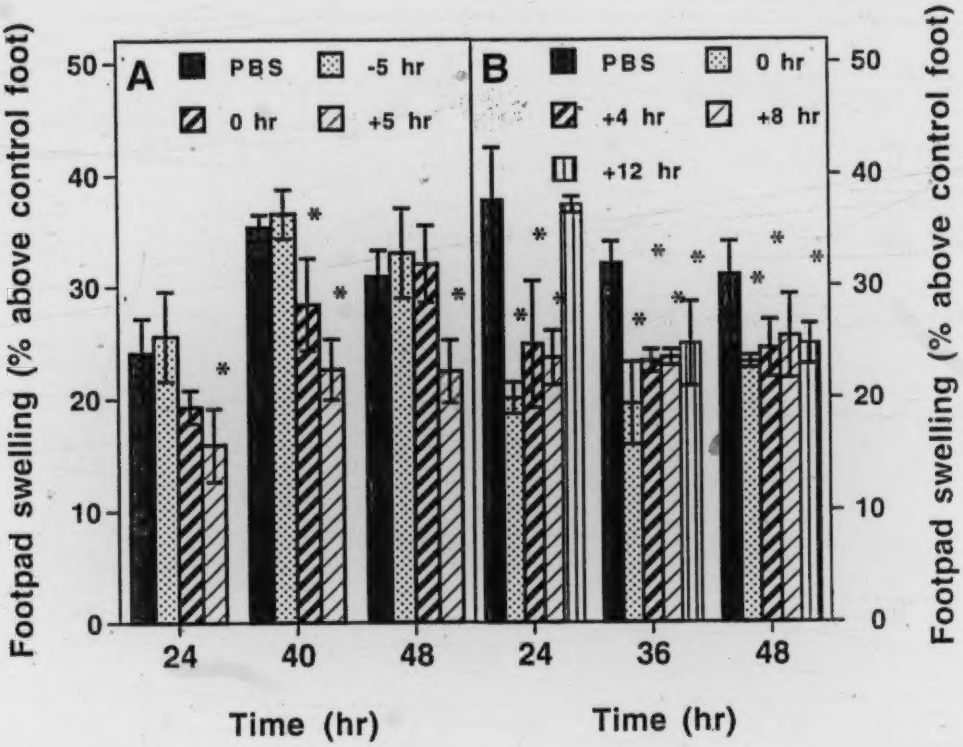


Figure 4.10. The time effect of IL-10 inhibition on DTH induced by M264-15 cells. DTH was induced by injecting 10^6 M264-15 cells into the left hind footpads of naive BALB/c mice. IL-10 (100µg/mouse) was administered by a single ip injection at different time points either before (-) or after DTH induction. Control mice received an equal volume of PBS at the same time as Th1 cell injection. Footpad swelling was measured at different time points indicated. Each bar represents the mean ± SD (n=3). * $p< 0.05$, compared to the control group.

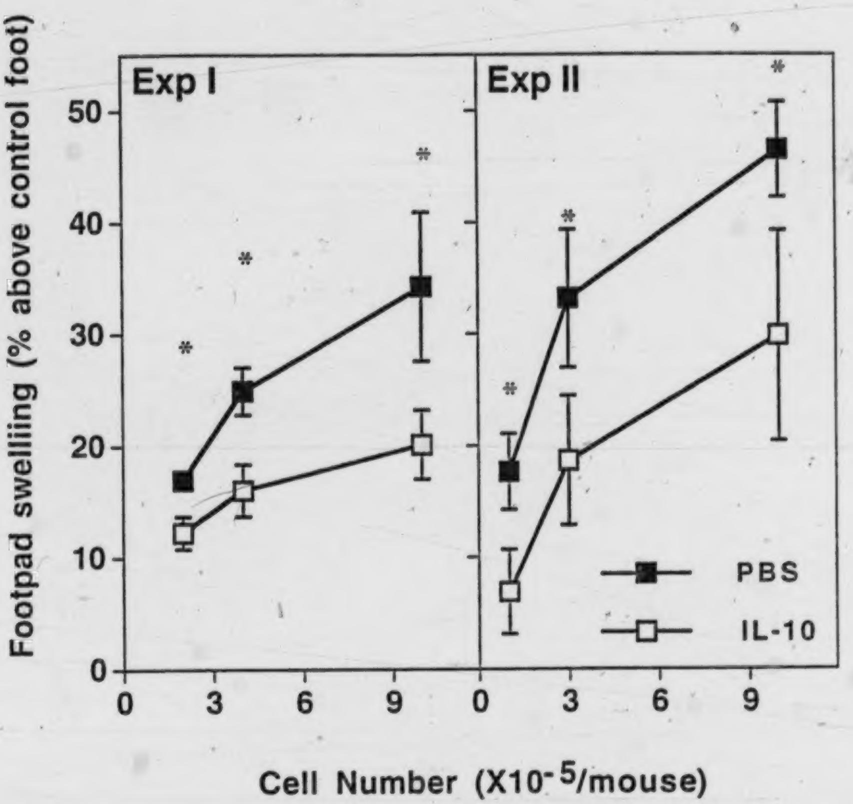


Figure 4.11. IL-10 inhibits DTH induced by different numbers of M264-15 cells. Different numbers of M264-15 cells were injected into the left hind footpads of naive BALB/c mice to induce DTH. For each Th1 cell dose, the animals received either 100µg IL-10 or an equal volume of PBS by a single ip injection at the same time as DTH induction. Footpad swelling was measured 24hr later. Each point represents the mean ± SD (n=3, Exp I; n=5, Exp II). * $p < 0.05$ compared to the control group.

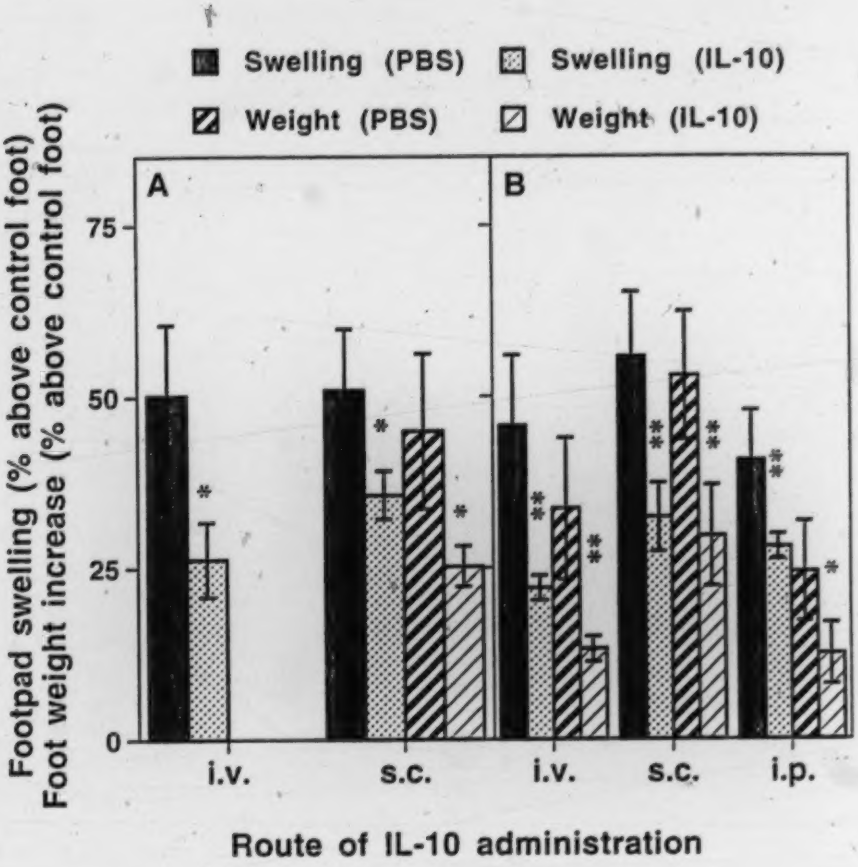


Figure 4.12. IL-10 administered by different systemic routes inhibits DTH induced by M264-15 cells. Each BALB/c mouse received 100µg (A, n=3) or 80 µg (B, n=5) IL-10 by a single iv, sc, or ip injection at the same time as DTH was induced by M264-15 cells (10^6/mouse). An equal volume of PBS was given by the corresponding routes as controls for each group. Twenty four hr later, either the footpad swelling or foot weight was measured. Each bar represents the mean ± SD. * $p < 0.05$, ** $p < 0.005$ compared to the control group.

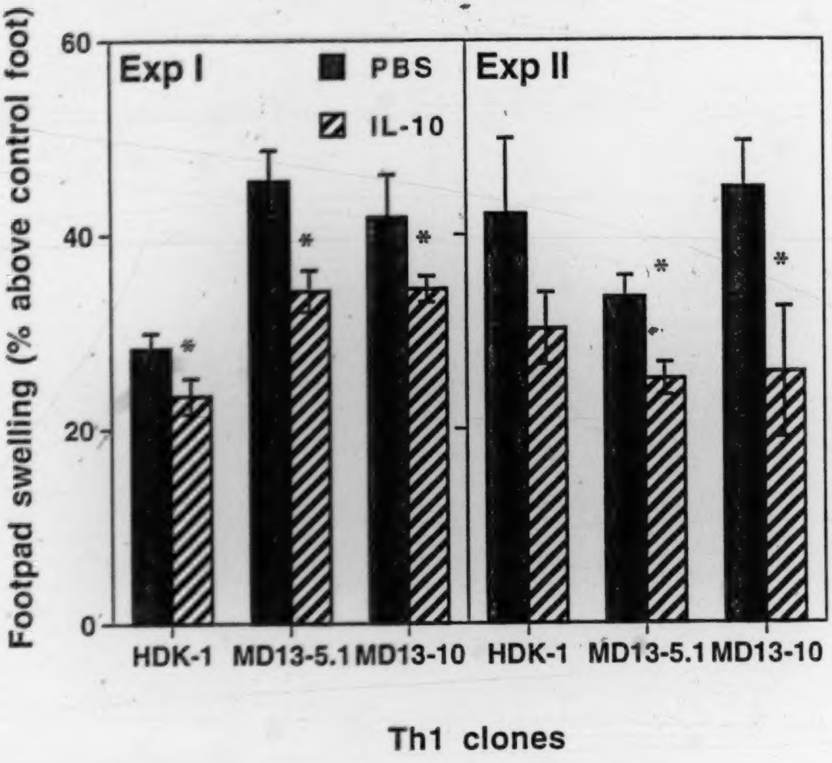
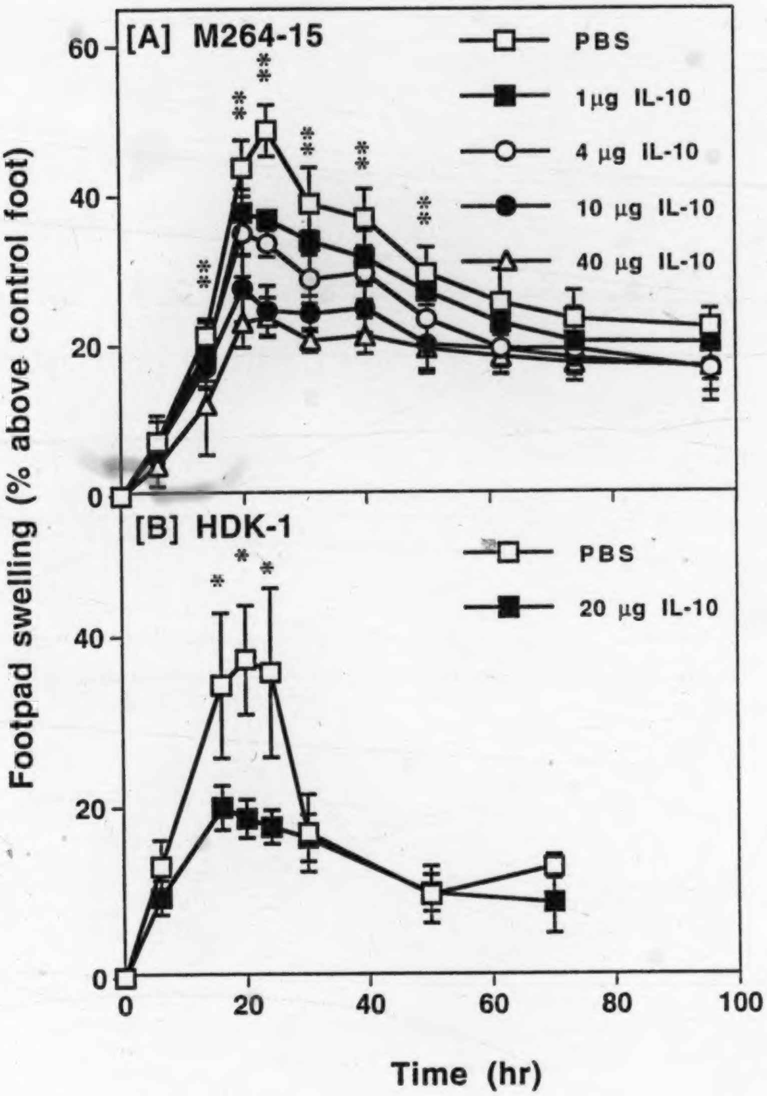


Figure 4.13. IL-10 inhibits DTH induced by other Th1 clones. DTH was induced by injecting 10^6 HDK-1 cells with 30 µg KLH, or 5×10^5 MD13-5.1 cells with 1.5×10^7 CRBCs, or 5×10^5 MD13-10 cells with 1.5×10^7 CRBCs into the left hind footpads of naive BALB/c mice. The right hind footpads of these mice were injected with equal numbers of the corresponding Th1 cells only. IL-10 (80µg/mouse) was given by a single ip injection, while the control mice received an equal volume of PBS. Footpad swelling was measured 24 hr after DTH induction. Each bar represents the mean ± SD (n=3). $^*p<0.05$, compared to the control group.


[A] M264-15
PBS
1µg IL-10
4 µg IL-10
10 µg IL-10
40 µg IL-10
[B] HDK-1
PBS
20 µg IL-10
Footpad swelling (% above control foot)
0
20
40
60
0
20
40
60
80
100
Time (hr)

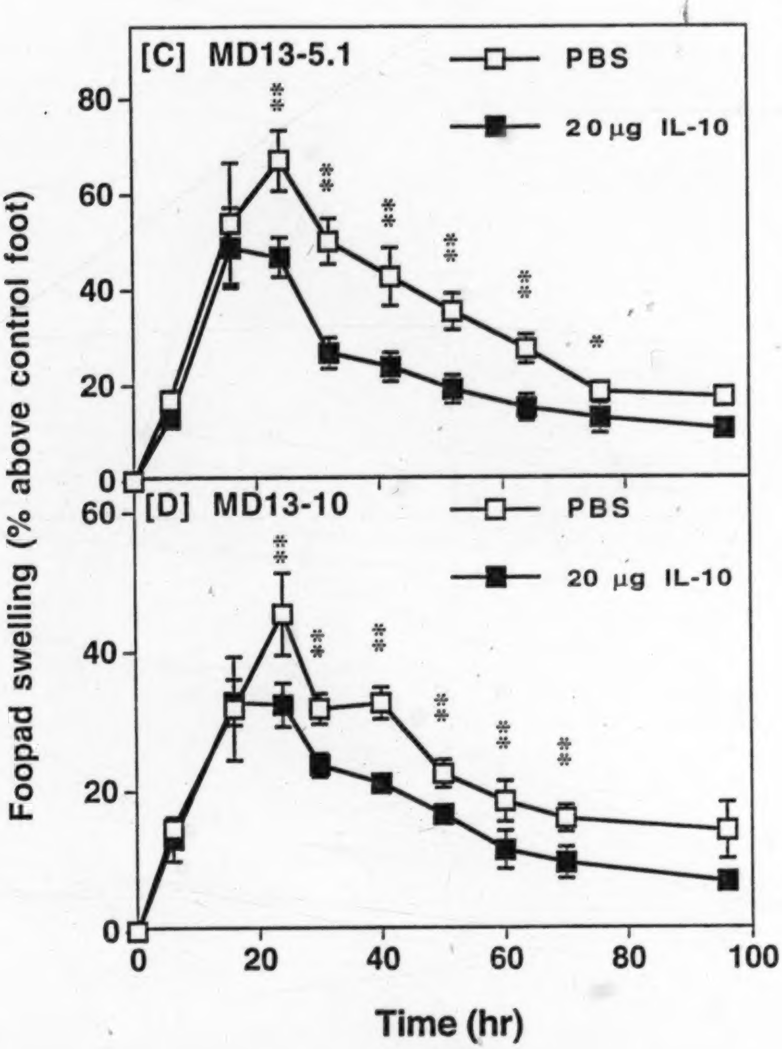


Figure 4.14. Time course analysis of the IL-10 suppression of DTH induced by Th1 clones. Footpad DTH was induced in naive BALB/c mice by the following Th1 clones and their antigens: 10^6 M264-15 cells (A, n=5), 10^6 HDK-1 cells with 30 μg KLH (B, n=3), 5×10^5 MD13-5.1 cells with 1.5×10^7 CRBC (C, n=4), or 5×10^5 MD13-10 cells with 1.5×10^7 CRBC (D, n=5). IL-10 was given by triple ip injections with the total doses indicated. The control mice received equal volumes of PBS. Each point represents the mean ± SD. * $p < 0.05$, ** $p < 0.005$, compared to the control group.

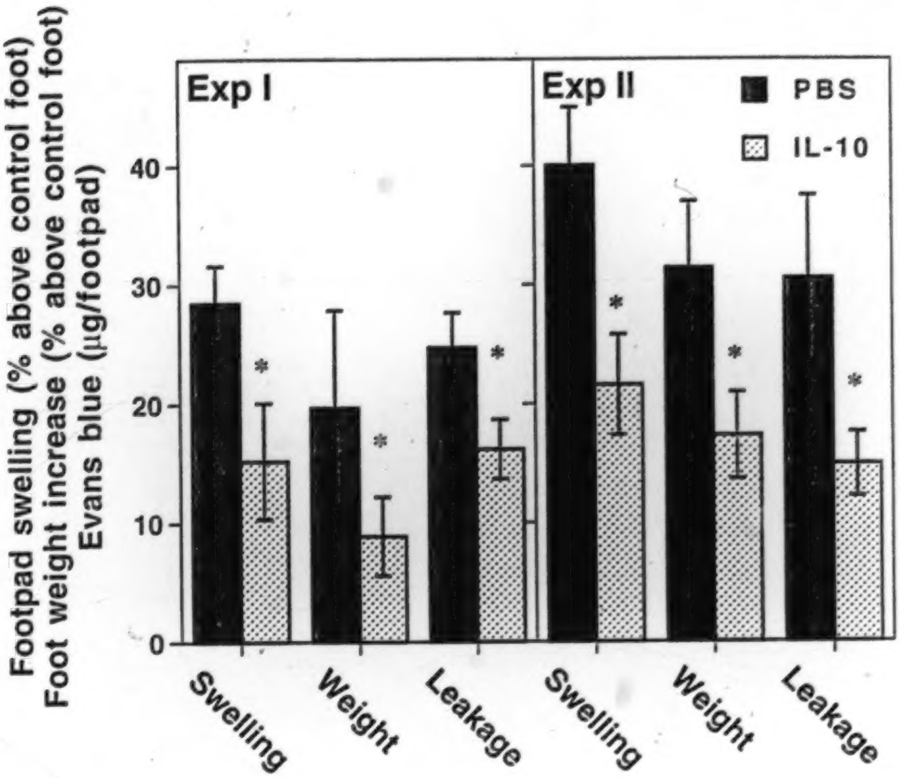


Figure 4.15. IL-10 inhibits tissue edema in DTH induced by M264-15 cells. DTH was induced by injecting 10^6 M264-15 cells into the left hind footpads of naive BALB/c mice. PBS or IL-10 (20µg/mouse total) was administered to these mice by triple ip injections. Eighteen hr after DTH induction, 0.1 ml of 1% Evans Blue dye in PBS was injected iv, 2 hr later the footpad swelling was measured, the mice were terminated and perfused. Their feet were collected and weighted. The Evans blue from each footpad was extracted and quantitated to represent the degree of vascular leakage. Each bar represents the mean ± SD (n=5). *$p<0.05$ compared to the control group.

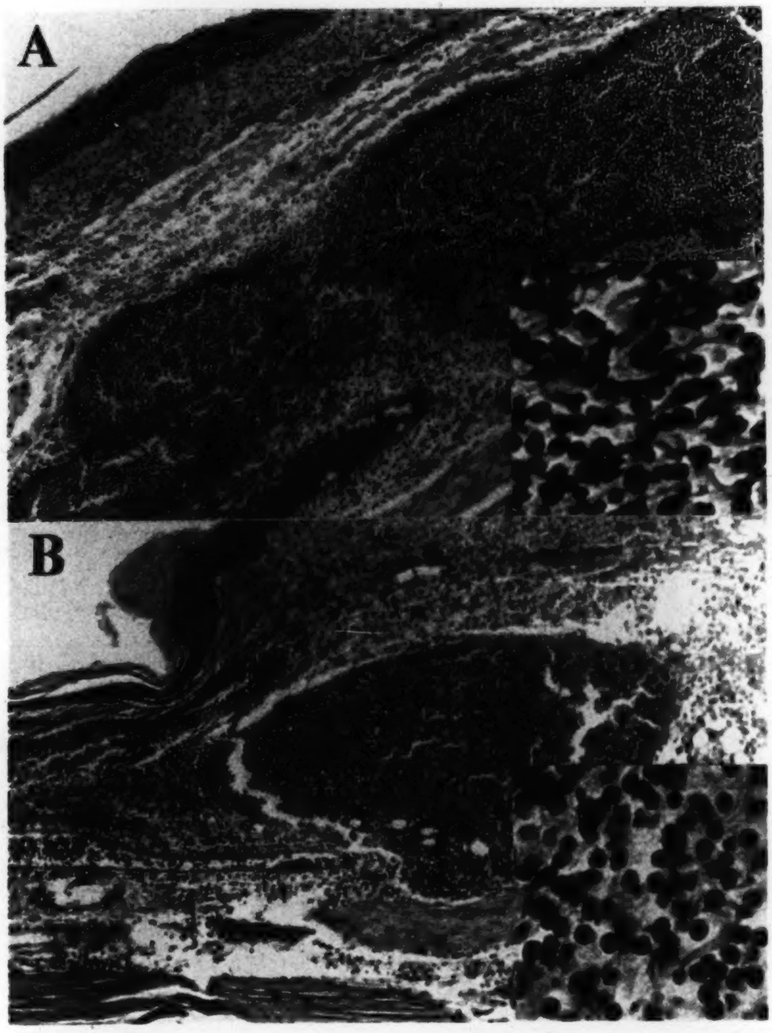


Figure 4.16. IL-10 dose not alter cellular infiltration in DTH induced by M264-15 cells. DTH was induced by injecting 10^6 M264-15 cells into the left hind footpads of naive BALB/c mice. IL-10 (100µg/mouse, B) was administered by a single ip injection at the same time as DTH induction. The control mice received an equal volume of PBS (A). Twenty-four hr after DTH induction, mice were sacrificed, their feet were collected, fixed with formaldehyde, sectioned and stained with Hematoxylin and Eosin (H-E).

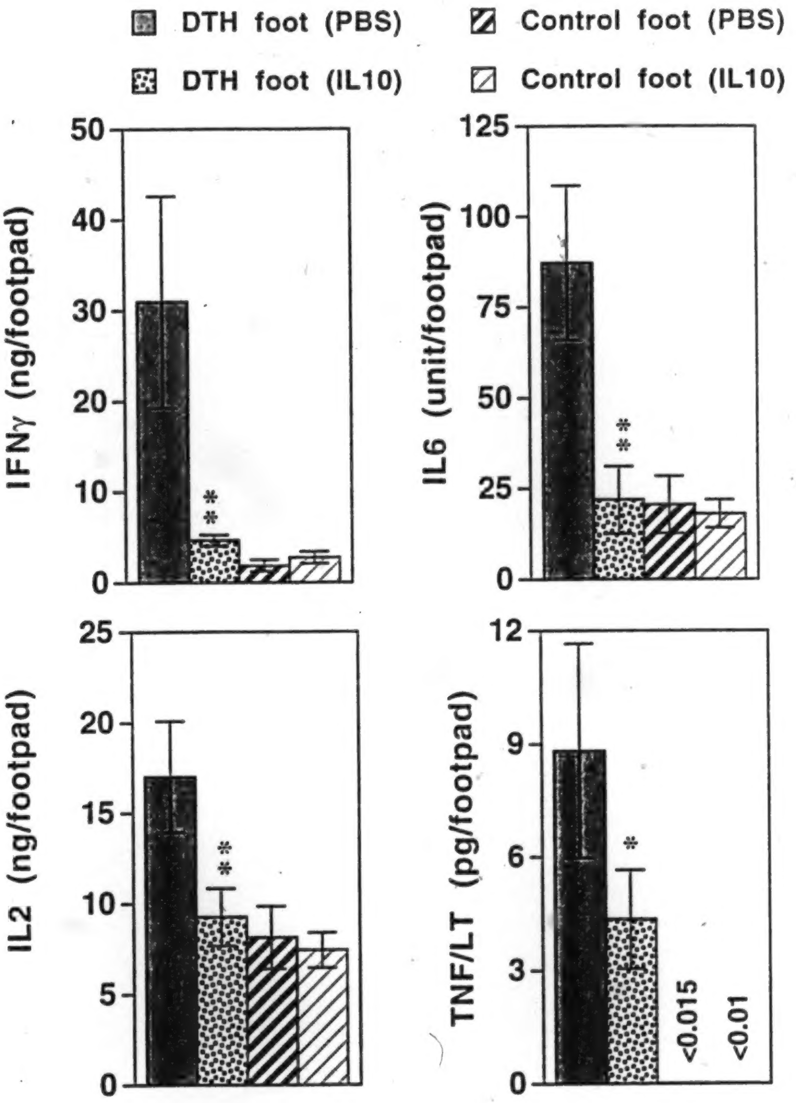


Figure 4.17. IL-10 inhibits cytokine levels in DTH footpads. BALB/c mice received 80μg/mouse IL-10 by a single iv injection at the same time as 10^6 M264-15 cells were injected into their left hind footpads to induce DTH. Twenty-four hr later, the footpads were collected, cut in HBSS and incubated on ice for 1 hr. The supernatants were then tested for cytokines by ELISA (IL-2, IL-6 and IFNγ) or a bioassay (TNF/LT). Each bar represents the mean ± SD (n=5). * $p<0.05$, ** $p<0.005$, compared to the control group.

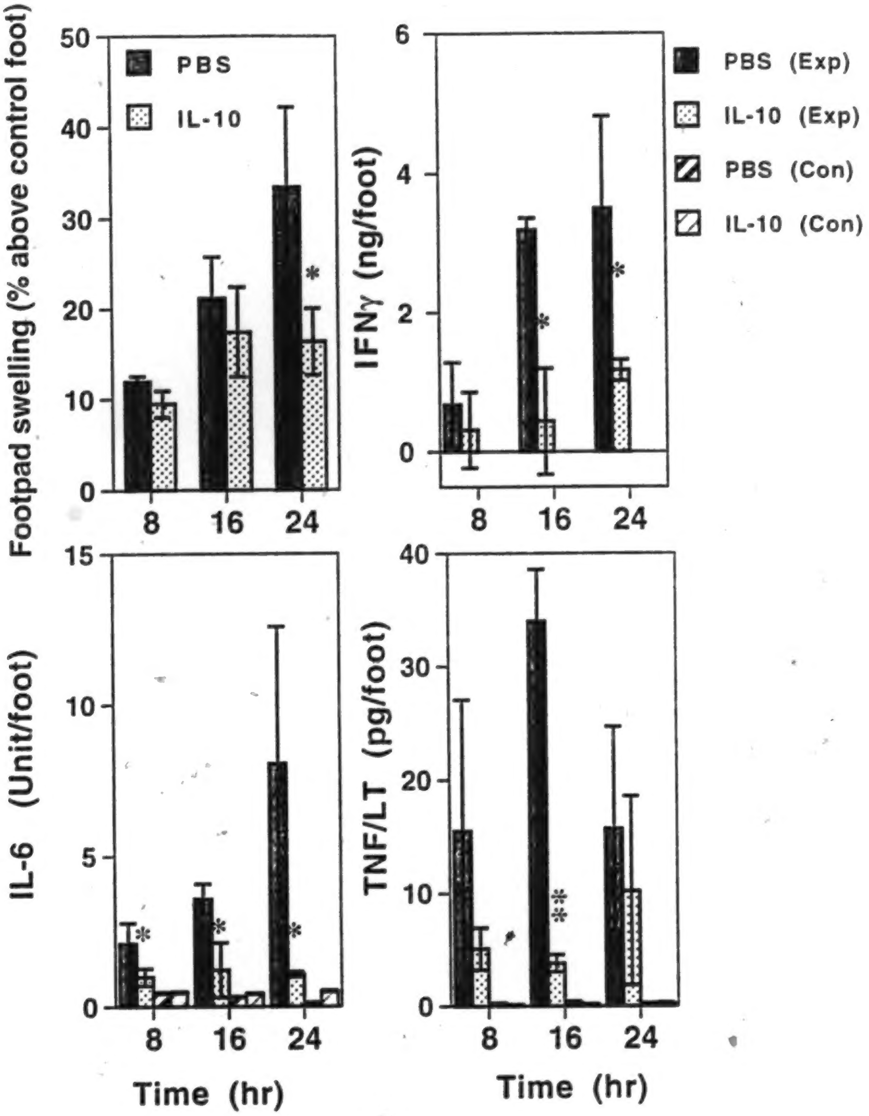


Figure 4.18. The time course of cytokine levels in DTH footpads, and the inhibitory effects of IL-10 on these cytokines. DTH was induced by injecting 10^6 M264-15 cells into the left hind footpads of BALB/c mice. PBS or a total dose of 20µg/mouse of IL-10 was administered by triple ip injections. Footpads were collected at 8, 16, and 24 hr after DTH induction. Cytokines were detected by ELISA (IL-6 and IFNγ) or a bioassay (TNF/LT) in the footpad extracts. Each bar represents the mean ± SD (n=3). $^*p<0.05$, $^{**}p<0.001$ compared to the control group.

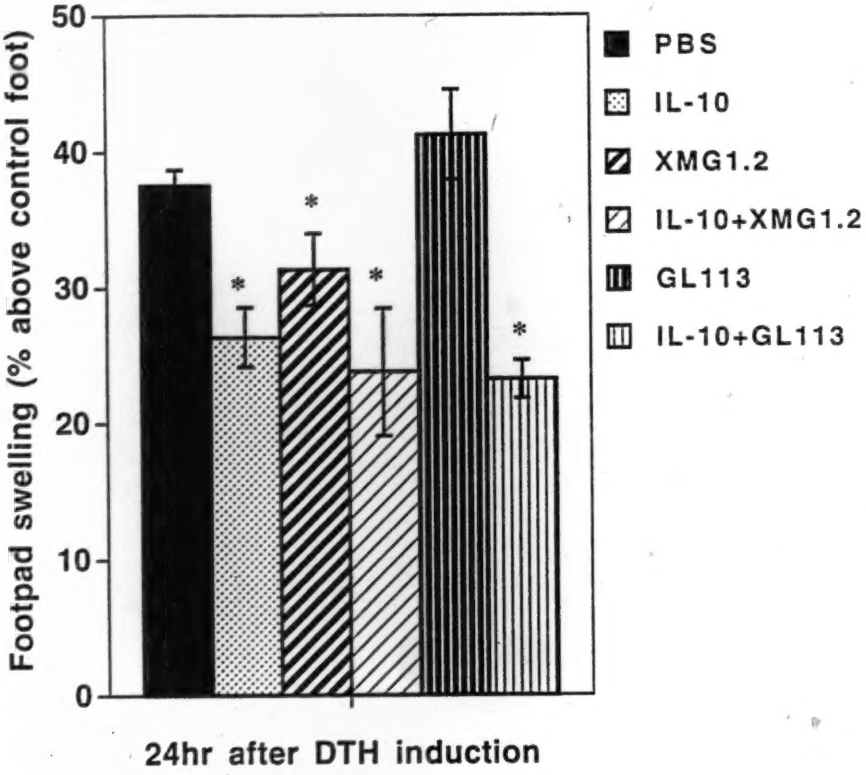


Figure 4.19. IL-10 does not show synergistic effect with anti-IFNγ in DTH inhibition. DTH was induced by injecting 10^6 M264-15 cells into the left hind footpads of BALB/c mice. These mice were treated with either 20μg of IL-10, or 1mg of anti-IFNγ antibody (XMG1.2), or 1mg isotype control antibody (GL113), or an equal volume PBS, or combinations of the above reagents as indicated. IL-10 was given by triple ip injctions at 0, 8 and 16 hr after T cell injection, while antibodies by a single ip injection at the same time as DTH induction. Footpad swelling was measured 24 hr after DTH induction. Each bar represents the mean ± SD (n=3). *$p<0.05$ compared to PBS or GL113 treated groups

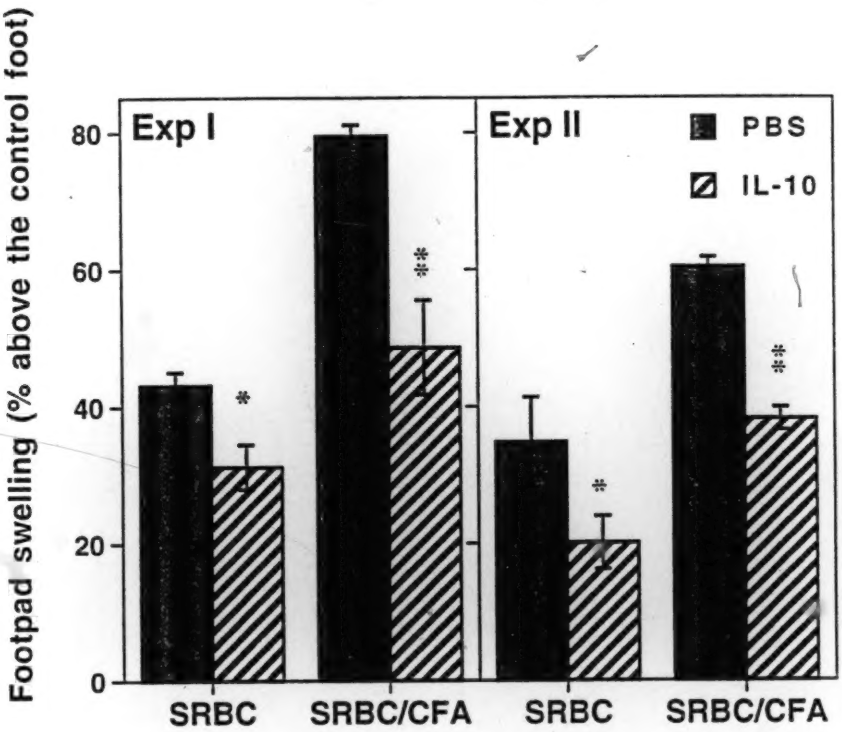


Figure 4.20. IL-10 inhibits the effector stage of DTH induced by priming and challenging with SRBCs. BALB/c mice were immunized sc with 10^8 SRBCs either with or without CFA. Fourteen d later, the mice were challenged with $2x10^6$ SRBCs in their left hind footpads, the right hind footpads of these mice receivd an equal volume of PBS as controls. IL-10 (100µg/mouse) was administered by a single ip injection at the same time as the SRBC challenge. Footpad swelling was measured 24 hr later. Each bar represents the mean ± SD (n=3). * $p< 0.05$, ** $p< 0.005$, compared to the control group.

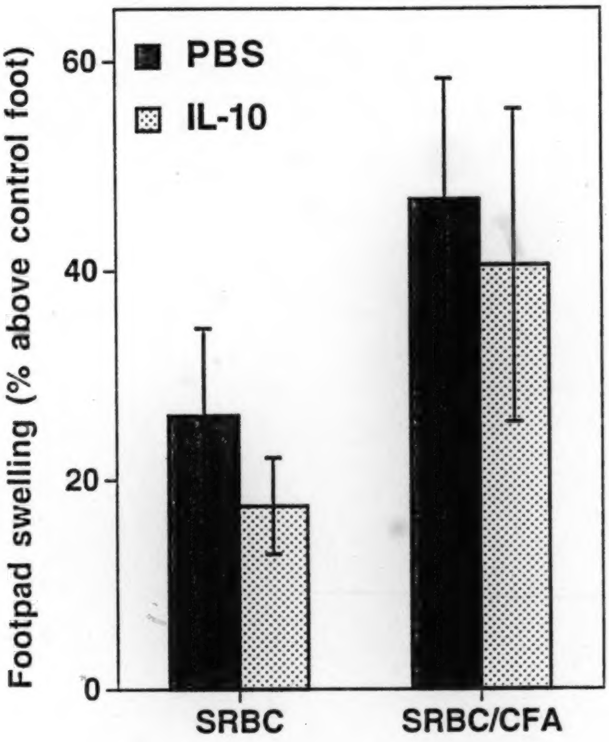


Figure 4.21. Smaller amounts of IL-10 administered by triple i.p. injections did not significantly inhibit DTH induced by SRBCs. BALB/c mice were immunized sc with 10^8 SRBC either with or without CFA. Fourteen d later, DTH was induced by injecting $2x10^6$ SRBC into the left hind footpads of the primed mice, and an equal volume of PBS was injected into the right hind footpads of these mice as controls. IL-10 (total 20µg/mouse) was administered by triple ip injections at 0, 8, and 16 hr after DTH induciton. Footpad swelling was measured at 24 hr. Each bar represents the mean ± SD (n=5).

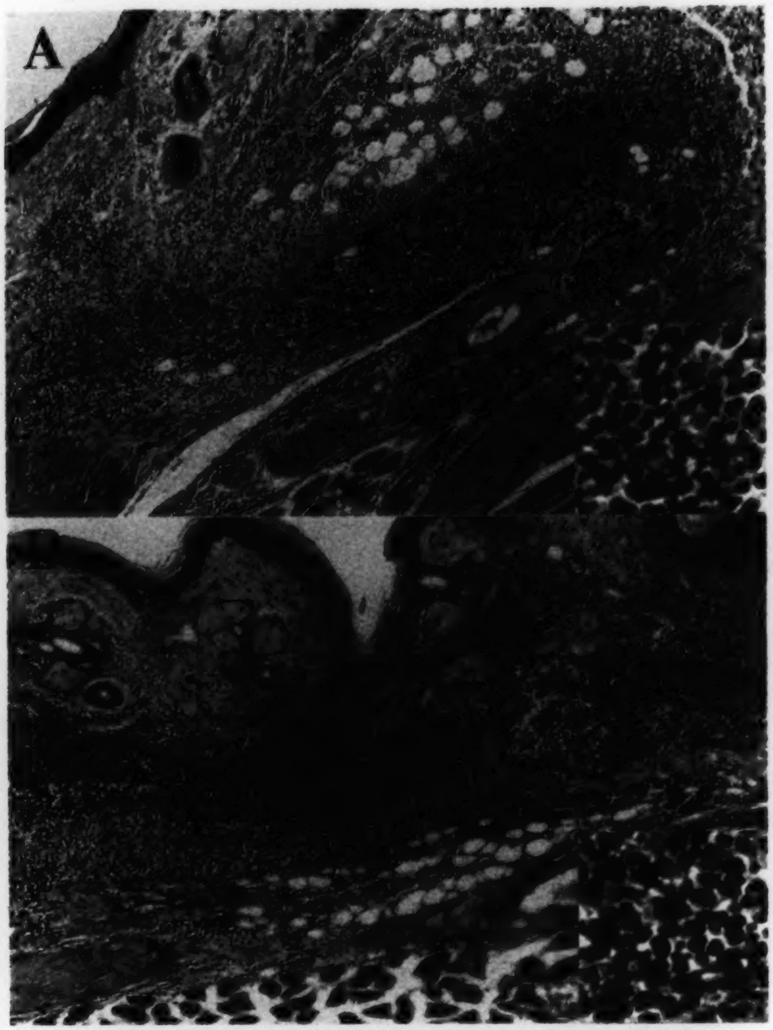


Figure 4.22. IL-10 does not alter cellular infiltration in SRBC-induced DTH. BALB/c mice were immunized with 10^8 SRBC with CFA, 14 d later DTH was induced by challenging these mice with 2x10^6 SRBC in their left hind footpads. IL-10 (100μg/mouse, B) was administered by a single ip injection at the time of antigen challenge, while the control mice received an equal volume of PBS (A). Twenty-four hr after the Ag challenge, mice were sacrificed, and their feet were cut off, fixed with formaldehyde, sectioned and stained with Hematoxylin and Eosin (H-E).

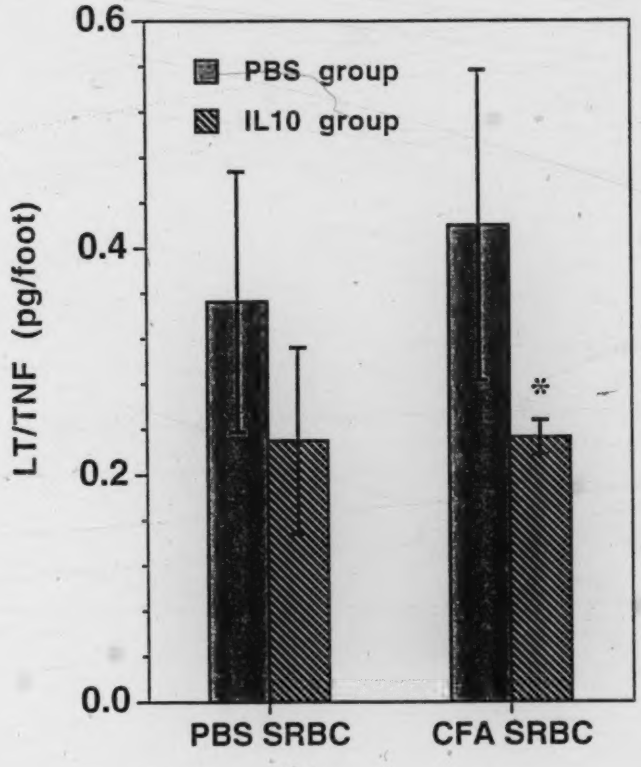


Figure 4.23. IL-10 inhibits levels of TNF/LT in SRBC DTH footpads. BALB/c mice were immunized sc with 10^8 SRBC with or without CFA. Fourteen d later, the mice were challenged by injecting 2×10^6 SRBCs into their left hind footpads. IL-10 (100µg/mouse) was given by a single ip injection at the time of SRBC challenge. Footpads were collected 24 hr later and cut in HBSS. TNF/LT was detected from the footpad extracts by a bioassay. Each bar represents the mean ± SD (n=3). * $p<0.05$, compared to the control group.

Table 4.1

Purification of Mouse rIL-10

	Total protein (mg)	Total IL10 U (ELISA)	IL10 specific activity (U/mg)	Accumulated fold of enrichment	Total recovery(%)
JE supernatant concentration	30.0	2.7×10^7	9×10^5		100
Blue 3GA column purification	6.9	2.3×10^7	3.3×10^6	3.7	85
Mono Q column purification	2.8	1.1×10^7	3.9×10^6	4.4	40

Table 4.2

IL-10 inhibits cytokine levels in the DTH footpads

Exp#	Cytokines	PBS(exp)	IL-10(exp)	PBS(con)	IL-10(con)	p*
Exp I	IL-2	9.09 ± 3.69	8.8 ± 1.45	3.6 ± 1.52	4.17 ± 3.58	
	IL-6	NDT	NDT	NDT	NDT	
	IL-10	24 ±5.2	19 ±5.2	8 ± 3.8	10 ± 5	
	IFNγ	18.36 ± 5.7	3.1 ± 1.8	-0.57 ±0.24	-0.82 ± 0.34	0.005
	TNF/LT	5 ± 1.2	3 ± 0.34	0	0	0.02
Exp II	IL-2	ND	ND	ND	ND	
	IL-6	ND	ND	ND	ND	
	IL-10	NDT	NDT	NDT	NDT	
	IFNγ	108 ±31.3	32.6 ± 5.5	0.88 ± 2.4	-0.7 ±0.4	0.002
	TNF/LT	3 ± 0.7	1 ± 0.24	0	0	0.001
Exp III	IL-10	156 ± 40.7	77 ± 30.2	85 ± 18	67 ± 31.7	
	Other cytokine values shown in Figure 4.17					

DTH was induced by injecting M264-15 (10^6cells/mouse) into the left hind footpads (exp) of naive BALB/c mice, the right hind footpads of these mice received an equal volume of PBS as controls (con). IL-10 (80μg/mouse) was injected sc (Exp I), ip (Exp II) or iv (Exp III), while the control mice received an equal volume of PBS with corresponding administration routes. The footpads were collected 24 hr after DTH induction, digested with collagenase and DNase (Exp I, II) or minced in HBSS (Exp III) solution at 4C. The supernatant was collected and the cytokines were detected by either ELISA (IL-2, IL-6, IL-10 and IFNγ) or bioassay (TNF/LT)

NDT: undetectable; ND: not done

*The cytokine values from the IL-10 treated footpads were compared to those from the control footpads. Significance was assumed at $p<0.05$ analyzed by a two-tail student's T test.

Chapter V

CD8 Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo, but induce similar footpad DTH

(Most of the data presented in this chapter have been published in articles entitled "CD8 Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar footpad inflammatory reactions" by Li Li, Subash Sad, David Kagi and Tim R. Mosmann in *J. Immunol.* (158:4152-4161) and "Cytokine-deficient CD8+ Tc1 cells induced by IL-4: Retention of inflammation and perforin-and Fas-cytotoxicity but compromised long-term killing of tumor cells" by Subash Sad, Li Li and Tim R. Mosmann in *J. Immunol.* (159: July 15 issue)

A. Introduction and rationale

Either CD4 or CD8 T cells can dominate a DTH response depending on the MHC restrictions of the antigens (Liew, 1982; Kundig et al., 1992). CD8 T subsets mediate DTH reactions to MHC class I restricted antigens, and are especially important in DTH toward infections by some viruses (Doherty et al., 1990; Leung and Ada, 1980) and other intracellular microbes (Muller, 1991; Muller et al., 1992; Goossens et al., 1992; Mody et al., 1994). In these infections, the DTH responses mediated by CD8 T cells either provide protection to the host, or are responsible for the pathogenesis of the disease. Therefore studies on the CD8 T cell mediated DTH could help us to understand the disease progress and to control the infections.

Similar to CD4 T cells, naive CD8 T cells can also differentiate into at least two subsets of effector cells with distinct cytokine patterns: Tc1 cells secrete a Th1-like cytokine pattern, including IL-2 and IFNγ, while Tc2 cells produce Th2 cytokines, including IL4 and IL5. These Tc1 and Tc2 cells were stable phenotypes that were not interchangeable (Sad et al., 1995). But when treated with IL-4, the cytokine production of mature Tc1 cells was dramatically decreased, and their ability to secrete IL-2 was totally lost (Sad and Mosmann, 1995). Thus, they were called the cytokine-deficient Tc1 cells. Due to the lack of IL-2 production, the autonomous proliferation of the cytokine-deficient Tc1 cells was affected. However their short term functions, such as perforin- and Fas-mediated cytotoxicity, were similar to those of normal Tc1 cells.

Local adoptively transferred DTH, induced by injecting primed T cells and their Ags into naive mouse footpads, represents the effector stage of the DTH process. The reaction presents a similar time course and histological appearance to DTH induced by direct antigen challenge of primed animals (Bianchi et al., 1981; Scovern and Kantor, 1982). Therefore it is often used to test the ability of certain cell types to induce DTH. In this model, resting Th1 but not Th2 clones induced footpad DTH, which was partially regulated by IFNγ (Cher and Mosmann, 1987; Fong and Mosmann, 1989). When pre-activated, Th2 populations were able to mediate IL-4-dependent DTH-like inflammation (Muller et al., 1993). The cellular infiltration of the Th2 induced DTH was similar to that induced by Th1 cells, but the peak reaction was significantly earlier (Muller et al., 1993). These results suggest that Th1 cells are more active in DTH induction, but Th2 cells also have the potential to induce DTH-like inflammation through different mechanisms. A CD8 T cell clone could also induce adoptively transferred footpad DTH (Lin and Askonas, 1981), however the cytokine pattern of this clone is not known.

As CD8 T cells are important regulatory cells of DTH responses in many diseases, and CD4 Th1 cells induce DTH more effectively than Th2 cells (Cher and Mosmann, 1987 and Figure 5.1), the potential abilities of Tc1 and Tc2 cells to induce DTH were tested in this study using the adoptively transferred DTH model. As DTH induced by Th1 and Th2 cells can be partially inhibited by anti-IFNγ and anti-IL-4 Abs, respectively, the Th1 and Th2 characteristic cytokines can be important regulatory factors of CD4 T cell-induced DTH. To study the cytokines potentially involved in the DTH induced by CD8 T cells, the cytokine levels were measured from the tissue extracts of the footpads injected with Tc1 or Tc2 cells. Furthermore using IL-4 and IFNγ blocking Abs, and cytokine-deficient Tc1 cells, the regulatory roles of IL-4 and IFNγ during Tc1 and Tc2 induced responses were addressed.

This project provides information about the contribution of the Th1- or Th2-like cytokine-producing CD8 T subsets to DTH responses, the characteristics of the DTH reactions, the potential cytokines involved and their regulatory roles.

B. Results

Both Tc1 and Tc2 cells adoptively transferred footpad DTH.

Tc1 and Tc2 cells were generated from naive CD8 T cells ($H2^b$) with allo-antigen ($H2^d$) stimulation in the present of IL-12 with anti-IL-4, and IL-4 with anti-IFNγ, respectively (Sad et al., 1995). Seven d after cell culture, an aliquot of these cells was stimulated with either Con A or antigen for 24 hr, and cytokines were measured from the supernatants to confirm the cytokine profiles of the T cells. Another aliquot of each type of T cell was stained for CD4 and CD8, and surface antigen expression was evaluated by FACScan. Figure 5.2 showed the cytokine patterns and phenotypes of d 7 Tc1 and Tc2 cells from a typical experiment. Usually, after checking the phenotypes and cytokine patterns of the CD8 T cells, d 10 Tc1 and Tc2 cells were used for DTH experiments.

The ability of Tc1 and Tc2 cells to induce footpad swelling was first tested using different numbers of T cells. $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells were injected into the footpads of naive BALB/c mice ($H2^d$) at doses ranging from 0.2 to 2 million per mouse. Footpad swelling was monitored over a period of 4-5 d. Both Tc1 and Tc2 cells induced cell dose-dependent, delayed footpad swelling (Figure 5.3) with similar kinetics and magnitude. Significant footpad swelling started as early as 4 hr after the cell injection, and the peak reaction appeared at 16-24 hr. Footpad swelling induced by either Tc1 or Tc2 cells was transient, and declined dramatically after 24 hr.

The ability of Tc1 and Tc2 cells to induce footpad swelling was also tested at clonal level. Tc1 and Tc2 clones were generated either by limiting dilution or by single T cell culture (add a single cell in each well by FACS sorting), and stimulated with allo-antigen ($H2^d$) in Tc1 or Tc2 differentiation conditions. The cloning efficiency was about

10% after the first 7 d stimulation. These cells were further restimulated every 7 d until $0.5\text{-}1 \times 10^6$ cells were obtained. Only about one third of the d 7 positive clones reached half to one million cells, and there were more Tc2 then Tc1 clones. The cytokine profile (Table I) of each clone was tested from Con A stimulated supernatants, and their CD8 expressions were checked by FACScan analysis. Most Tc1 and Tc2 clones induced significant footpad swelling in BALB/c mice 20-24 hr after T cell injection (Figure 5.4), suggesting that Tc1 or Tc2 cells alone were sufficient to induce the footpad reaction. Table I is a summary of the cytokine production and the footpad swelling induced by all the Tc1 and Tc2 clones obtained.

The T cell activation state required for the footpad reaction was then studied by comparing resting and activated Tc1 or Tc2 cells. Resting cells were obtained by maintaining the d 7 Tc1 and Tc2 cells in IL-2-containing medium for another 2 wk. These cells were smaller in size, incorporated less ^{3}H thymidine, and expressed no detectable cytokines in their supernatants (data not shown). Activated Tc cells were obtained by restimulating the resting T cells with APCs for 24 hr. These cells expressed cytokines in their supernatant. When equal numbers of resting and activated Tc1 or Tc2 cells were injected into the footpads of naive BALB/c mice, surprisingly they induced similar footpad swellings (Figure 5.5), except that activated Tc1 cells induced strong swelling slightly earlier (16 hr) than the resting Tc1 cells.

Footpad swelling induced by resting or activated Th1 and Th2 cells was also compared. Th1 and Th2 cells were generated from CD4 splenocytes of C57BL/6J ($H2^b$) mice with allo-stimulation (M12.4.1, $H2^d$) in the culture conditions of IL-12 with anti-IL-4, and IL4 with anti-IFNγ, respectively. The resting cells were obtained by maintaining d 7 Th1 and Th2 cells in IL-2 containing medium for an additional 2 wk, and the activated Th cells were obtained by restimulating the resting T cells with APCs for 24 hr. Both resting and activated Th1 cells induced footpad swelling, but the reaction induced by the activated cells was significantly stronger around the peak reaction. Resting Th2 cells showed lower activity in DTH induction than Th1 cells, however when pre-activated, these cells induced comparable footpad swelling to that induced by the activated Th1 cells (Figure 5.5), suggesting that Th2 cells may not be fully activated in vivo. In general, both Tc1 and Tc2 cells induced stronger footpad swelling than Th cells.

DTH induced by Tc1 and Tc2 cells was antigen-specific, and did not require host T cells.

The antigen specificity of the footpad reaction induced by Tc1 or Tc2 cells was tested using different strains of mice. Equal numbers of $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells were injected into the footpads of naive BALB/c ($H2^d$), C57BL/6 ($H2^b$) and CBA ($H2^k$) mice. Significant footpad swelling was induced only in BALB/c mice, but not in syngeneic C57BL/6 mice, nor in third party CBA mice (Figure 5.6). This result indicated that the footpad reaction induced by Tc1 and Tc2 cells was antigen specific.

Since the injected Tc1 and Tc2 cells ($H2^b$) were allogeneic to the BALB/c mice ($H2^d$), it is possible that the footpad reaction was caused by the allo-rejection response mounted by the host T cells. To ensure that the host immune system did not react against the injected T cells, C57BL/6 anti-$H2^d$ Tc1 and Tc2 cells were injected into BALB/c x C57BL/6, $H2^{d/b}$ F1 mice. Tc1 and Tc2 cells both induced significant footpad swelling in the F1 ($H2^{d/b}$) as in BALB/c mice (Figure 5.7).

The lack of contribution by host T cells was confirmed by using SCID mice, which do not have mature endogenous T cells, and normally do not reject allo-grafts. Both Tc1 and Tc2 cells induced similar footpad swelling in $H2^d$ SCID mice compared to BALB/c mice (Figure 5.8). This result suggested that host T cells were not required for the footpad swelling induced by Tc1 or Tc2 cells, therefore the footpad reaction was not likely to be due to the host allo-rejection response.

Tc1 and Tc2 cells induced similar edema during the footpad reaction

The tissue swelling of a DTH response is normally a combined result of cellular infiltration and edema. To determine if Tc1 and Tc2 cells induced similar edema during the footpad reaction, vascular leakage was measured using Evans Blue dye. Twelve hr after Tc1 or Tc2 cell injection, BALB/c mice were injected iv with Evans Blue. Six hr later, the mice were sacrificed, perfused, and the Evans Blue in the footpads was extracted and quantitated. Elevated amounts of Evans Blue were detected in both Tc1 and Tc2 injected footpads (Figure 5.9), suggesting that increased vascular permeability was induced during the footpad reaction mediated by these cells. Similar footpad swelling induced by Tc1 and Tc2 cells was accompanied by similar levels of vascular permeability as indicated by Evans Blue leakage.

Cellular infiltration in the footpads injected with Tc1 or Tc2 cells

Cellular infiltration is another important characteristic of DTH. Neutrophils and macrophages are the two major cell types that migrate into local tissue during DTH responses, therefore the kinetics of appearance of these two cell types were tested in the footpads injected with Tc1 or Tc2 cells. Immunohistochemical staining specific for Gr-1 (a surface marker for granulocytes; Hestdal et al., 1991) was performed on footpad sections collected at 6, 12 or 24 hr after T cell injection. Gr-1 positive cells were detectable as early as 6 hr, and the infiltrate was more intense at 12 hr and 24 hr with a similar appearance between Tc1 and Tc2 injected footpads. Figure 5.10 showed the representative tissue sections collected at 12 hr. Sections from Th1-injected footpad were stained for Gr-1 as positive controls, as granulocyte infiltration was dominant in footpad DTH induced by Th1 cells (Fong and Mosmann, 1989). Tissue sections from the RPMI injected control footpads were also stained for Gr-1 as negative controls (Figure 5.10).

Infiltrated macrophages were detected using anti-Mac-3 staining, which stains mainly activated macrophages (Ho and Springer, 1983). Mac-3 positive cells were not detected at 6 hr in either Tc1 or Tc2 injected footpads, but were present by 12 and 24 hr with similar intensities between Tc1 and Tc2 cell induced reactions. Figure 5.11 showed the representative tissue sections collected at 24 hr. Collectively both granulocytes and macrophages were infiltrated in the inflamed footpads injected with Tc1 or Tc2 cells, therefore, the footpad reaction induced by the Tc cells represented a DTH-like inflammatory response.

The extent of Tc1- and Tc2-induced cellular infiltration was compared by preparing single-cell suspensions from footpads collected 24 hr after Tc cell injection. The numbers of live cells recovered from Tc1 and Tc2 injected footpads were $(8.5 \pm 1.3)\times10^4$ and $(8.8 \pm 1.3)\times10^4$ cells respectively, while $(2.7 \pm 0.3)\times10^4$ and $(2.4 \pm 0.5)\times10^4$ cells were extracted from the corresponding control footpads. These recovered cells were then assessed by staining their cytospin preparations. Gr-1 staining was used to detect granulocytes in the cytospin preparations (Figure 5.12), and the positive cells were counted. Similar numbers of Gr-1$^+$ cells were detected from the Tc1 and Tc2 injected footpads (Figure 5.13a), and these cells constituted about 20% of the total cells recovered (Figure 5.13b). Although cells expressing high Gr-1 levels are mostly neutrophils, eosinophils can express low levels of Gr-1 (Hestdal et al., 1991). To distinguish neutrophils from eosinophils, the infiltrated cells were also stained with Methylene Blue and Eosin Y (Figure 5.14). The levels of neutrophils revealed by Eosin and Methylene Blue staining were similar to those of the Gr-1$^+$ cells in either Tc1 or Tc2 footpads (Figure 5.13), suggesting that the majority of the Gr-1^+ cells detected in the footpads were neutrophils. Thus, similar neutrophil infiltration was induced by Tc1 or Tc2 cells.

Macrophages were also detected in the cytospin preparations from both Tc1- and Tc2- injected footpads using anti-Mac-3 staining (Figure 5.15), and the positive cells were counted with all slides coded. At 24 hr after T cell injection, both Tc1 and Tc2 DTH footpads yielded large numbers of Mac-3^+ cells (Figure 5.16a) that constituted about 50% of the total recovered cells (Figure 5.16b). However there was again no detectable difference between Tc1 and Tc2 cell injections.

Th2 responses are often accompanied by increased eosinophil infiltration. To test if eosinophils also participate in DTH induced by Tc2 cells, the extents of infiltrated eosinophils were detected using Eosin Y and Methylene Blue staining of the extracted cells (Figure 5.14). Although eosinophils constitute less than 3% of the total blood leukocytes, 10-20% of the cells recovered from both Tc1 and Tc2 DTH footpads were eosinophils (Figure 5.17), indicating that a preferential eosinophil recruitment occurred in CD8 T cell mediated DTH. Furthermore, the numbers of infiltrated eosinophils were significantly ($p<0.05$, two-tailed student's t-test) larger in the Tc2 injected footpads, suggesting a possible contribution of the Tc2 cytokines: both IL-4 and IL-5 are eosinophil chemoattractants (Wang et al., 1989; Tepper et al., 1992; Dubois et al., 1994), and IL-5 is also an eosinophil survival factor (Stern et al., 1992).

The time course of the eosinophil appearance was studied in cytospin preparations from footpads collected at 12, 24 and 40 hr after Tc1 or Tc2 cell injections. In this experiment, the numbers of eosinophils infiltrating Tc1 or Tc2 injected footpads were similar at both 12 and 24 hr (Figure 5.18), while significantly ($p<0,05$, two-tailed student's t-test) more eosinophils were present in the Tc2 injected footpads at 40 hr after the cell injection. This result indicated that the Tc2 cytokine effect on eosinophil infiltration may be stronger at later time point of the DTH reaction.

Cytokine synthesis in vivo during DTH induced by Tc1 or Tc2 cells

The above results demonstrated that Tc1 and Tc2 cells induced similar footpad DTH despite producing different patterns of cytokines in vitro. To confirm that Tc1 and Tc2 cells continued to produce their characteristic cytokines while inducing similar footpad DTH, cytokine levels were measured in footpad extracts. Footpad DTH was induced in naive BALB/c mice with either Tc1 or Tc2 cells. Mice were terminated at 8, 16, and 24 hr after DTH induction, and tissue extracts were prepared from individual footpads. The levels of the Tc1 or Tc2 specific cytokines, such as IFNγ, IL-4 and IL-5, were measured by ELISA. Significant levels of IFNγ (Figure 5.19a) were detected in the footpads injected with Tc1 but not Tc2 cells, while IL-4 (Figure 5.19b) and IL-5 (Figure 5.19c) were found in the Tc2 but not Tc1 footpads. All these three cytokines reached high levels at 8 hr after the cell injections, suggesting a rapid activation of these cells in vivo. IFNγ was often low by 24 hr, whereas IL4 and IL5 levels remained high (Figure 5.19), and sometimes were still detectable at 40 hr after the T cell injection (data not shown). IL-2 and IL-10 were not detected in footpad extracts using ELISAs with assay sensitivities of 0.06 and 0.2 ng/ml respectively. No significant levels (compared to assay background) of cytokines were detected in the control footpads of any mice, suggesting that the Tc1 and Tc2 cytokines were produced by the injected T cells.

Cytokines directly involved in inflammation, such as IL-6 and TNF/LT, were also measured in the footpad extracts. Besides T cells, these cytokines can be produced by activated macrophages, mast cells and/or endothelial cells during inflammation, thus, the elevation of their levels in the tissue often indicates an ongoing inflammatory response.

Similar levels of IL-6 (detected by ELISA, Figure 5.20a) and TNF/LT (by bioassay, Figure 5.20b) were detected in both Tc1 and Tc2 injected footpads before or at the peak DTH reaction. The relationship between the in vivo levels of these cytokines and the footpad swelling was analyzed. A positive correlation, particularly for TNF/LT was found between the levels of these cytokines in the footpads and the footpad swelling at either 16 or 24 hr (Figure 5.21). This correlation suggested a direct participation of these cytokines in the footpad DTH responses.

$H2^b$-anti-$H2^d$ Tc1 and Tc2 cells induced significant footpad swelling in the F1 mice of BALB/c x C57BL/6J ($H2^{d/b}$) as well as in BALB/c ($H2^d$) mice, but not in the syngeneic C57BL/6J ($H2^b$) mice (Figure 5.7). In correlation, the Tc1 and Tc2 specific cytokines were also detected in the F1 mouse footpads, except that all cytokine levels were generally lower than those in the BALB/c mice (Figure 5.22), possibly due to reduced levels of the target MHC in the F1 mice. There were no significant levels of either Tc1 or Tc2 cytokines in the extracts of the T cell injected footpads of syngeneic C57BL/6 mice (Figure 5.22). These results suggested that the Tc1 and Tc2 cytokines detected in DTH footpads were produced upon antigen-specific activation of the corresponding T cells. Elevated levels of IL-6 and TNF/LT (Figure 5.23) were also detected in footpads of BALB/c and F1 mice, which agreed with the footpad DTH induced in these mice.

Collectively, these results demonstrated that upon antigen-activation, Tc1 and Tc2 cells produced and retained their in vitro cytokine patterns in the injected footpads, therefore, similar inflammatory responses, accompanied with similar secondary inflammatory cytokine production, were induced in both Tc1 and Tc2 cytokine environments.

Anti-cytokine antibody treatment of DTH induced by Tc1 and Tc2 cells

DTH induced by Th1 and Th2 cells was positively regulated by IFNγ and IL-4 respectively. To test if IFNγ and IL-4 are also important mediators for DTH induced by Tc1 or Tc2 cells, antibodies to these cytokines were administered 1 hr before the DTH induction. None of the Ab treatment significantly inhibited DTH induced by either Tc1 or Tc2 cells (Figure 5.24). This result indicated that either the antibody block was not complete, or unlike the DTH induced by Th1 and Th2 cells, IFNγ and IL-4 may not be the critical regulatory factors for DTH induced by Tc1 or Tc2 cells.

Lack of correlation between Tc1 cytokine synthesis and DTH induction

IFNγ is often associated with DTH responses. However the failure to inhibit Tc1-induced DTH with anti-IFNγ, and the ability of Tc2 cells, which do not produce IFNγ at all, to induce DTH, all suggested that this cytokine may not be critical for DTH induced by CD8 T cells. This hypothesis was further tested using the cytokine-deficient Tc1 cells ($Tc1^-$), which were obtained by incubating mature Tc1 cells with IL-4. When an equal number of Tc1 and the $Tc1^-$ cells were injected into BALB/c mouse footpads, similar footpad swelling was induced by these two types of cells with similar kinetics (Figure 5.25).

To test the cytokine levels in the footpads injected with Tc1 and $Tc1^-$ cells, mice were terminated 22 hr after DTH induction, and cytokines were measured in the footpad extracts. Although the $Tc1^-$ cells only produced about one eighth of the IFNγ produced by normal Tc1 cells (Figure 5.26b), a similar magnitude of footpad swelling was induced by these two types of cells (Figure 5.26a), suggesting a lack of correlation between the in vivo IFNγ synthesis and the DTH induction. Similar levels of the secondary cytokine, IL-6, were induced by these two types of cells, which agreed with the similar footpad swelling they induced.

The correlation between IFNγ and DTH induction was also tested using different doses of Tc1 and Tc1⁻ cells. Tc1⁻ cells induced cell dose-dependent footpad swelling with similar magnitudes to those induced by same numbers of normal Tc1 cells (Figure 5.27), although the Tc1⁻ cells produced lower IFNγ than Tc1 cells in vivo at all the corresponding cell doses. Furthermore, the in vivo IL-6 and TNF/LT levels were positively correlated with the cell dose-dependent footpad swelling (Figure 5.27). As Tc1 cells don't produce IL-6 in vitro (Figure 5.2), IL-6 detected in the footpads was most likely to be secondary. The similar levels of TNF detected in Tc1 and Tc1⁻ injected footpads suggested that the TNF in the footpads was also secondary, as Tc1⁻ produced much less TNF than normal Tc1 cells in vitro. Collectively, these results demonstrated that the in vivo IFNγ levels were not correlated with the footpad swelling, therefore, either the in vivo IFNγ concentration needed for DTH induced by Tc1 cells was low, or IFNγ was not as critical in Tc1 DTH as it is in the Th1 DTH.

Footpad swelling was induced in syngeneic mice by injecting Tc1 or Tc2 cells together with APCs.

Adoptively transferred DTH is usually induced by injecting T cells with their appropriate antigens into syngeneic mice. The experiments shown thus far have involved injecting allospecific T cells into mice in which all tissues bear the target antigen. We have also injected allo-specific Tc1 and Tc2 cells into syngeneic mice with allo-APCs to test their ability to induce DTH. The control feet of these mice were injected with Tc1 and Tc2 cells alone respectively. Two kinds of allo-APCs ($H2^d$) were used, the B hybridoma M12.4.1 and the macrophage cell line J774. When M12.4.1 was used as

APCs, Tc1 cells induced stronger footpad swelling than Tc2 cells, while when J774 was used, similar footpad swelling was induced by Tc1 and Tc2 cells (Figure 5.28). However the magnitude of the footpad swelling was generally less than that induced in the target mice (BALB/c), and the peak reaction was earlier.

Systemically transferred Tc1 or Tc2 cells induced only marginal footpad swelling

Local adoptively transferred footpad DTH only represents the effector stage of a DTH process, as antigen-specific T cells are injected directly into the footpad rather than being recruited by the normal DTH-initiating events. Since T cell homing to an inflamed site is critical for a DTH response, a systemically transferred DTH model was used to test the ability of Tc1 and Tc2 cells to induce DTH. $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells (10^7cell/mouse) were injected into syngeneic C57BL/6 ($H2^b$) mice iv, these mice were then challenged with J774 cells in their left hind footpads, with their right hind footpads injected with RPMI as controls. Footpad swelling was then monitored every 8-12 hr for days. The footpad swelling induced by antigen challenge was stronger than that of the controls, but it was often too weak to be significant compared to the control feet (Figure 5.29). These results suggested that either the challenged allo-cells were not able to efficiently initiate a inflammatory response, or alternatively the systemically injected Tc1 and Tc2 cells cannot migrate efficiently to a local inflammation site.

Both Tc1 and Tc2 cells preferentially migrated to inflamed sites

T cell homing to an inflamed site is critical for a DTH response. Since allo-reactive Tc1 and Tc2 cells did not efficiently induce footpad swelling when transferred systemically into syngeneic mice, the ability of Tc1 and Tc2 cells to migrate from the circulation into the inflammatory tissue was tested as an event that was separated from footpad swelling. Inflammation was induced by injecting either Tc1 or Tc2 ($H2^b$-anti-$H2^d$) cells together with cells bearing the target MHC ($H2^d$) into the left hind footpad of C57BL/6J ($H2^b$) mice. Six hr after the induction of the inflammation, ^{51}Cr labeled Tc1 or Tc2 ($H2^b$-anti-$H2^d$) cells (10^7/mouse) were injected into these mice iv. After another 6 hr, the experimental and control feet were collected and the radioactivity measured. In all cases, significantly higher levels of radioactivity were detected in the feet with an ongoing inflammation than in the control feet (Figure 5.30), suggesting that both Tc1 and Tc2 cells can preferentially migrate into a tissue in which inflammation has been initiated. Similar Tc1 migration was detected in Tc1 and Tc2 cell induced inflammation. Tc2 migration was slightly lower than Tc1 cells in three independent experiments, but again migration into either Tc1- or Tc2-induced inflammatory sites was similar. Thus, neither Tc1 nor Tc2 cells showed any preference for migrating into an inflammatory site induced by either cytokine pattern. Calculated by the radioactivity, it was found that less than 1% ($<10^5$ cell/foot) of the iv injected cells had migrated into the inflamed feet, and this result agreed with the earlier findings that less than 4% of the iv injected cells remained in circulation 10 min. after transfer (Turk, 1962). As both Tc1 and Tc2 can also induce footpad DTH after local adoptive transfer, together, these results suggest that Tc1 and Tc2 cells have the potential to mediate DTH during in vivo responses.

C. Summary of the results:

1) $H2^b$-anti-$H2^d$ Tc1 and Tc2 cells induce similar footpad swelling, edema, and neutrophil/macrophage infiltration in $H2^d$ or $H2^{d/b}$ mice. Furthermore, Tc2 cells cause more eosinophil infiltration.

2) The footpad DTH induced by Tc1 and Tc2 cells is antigen-specific, and does not require endogenous T cells.

3) Tc1 and Tc2 cells retained their in vitro cytokine profiles in the injected footpads, and induced similar secondary inflammatory cytokines, indicating that similar inflammation can be induced in both Tc1 and Tc2 cytokine environments.

4) Anti-IFNγ or anti-IL-4 treatment has little effect on the DTH induced by either Tc1 or Tc2 cells, suggesting that these cytokines may not be critical.

5) Cytokine deficient Tc1 cells produced much less IFNγ in vitro and in vivo, but induced similar footpad DTH as normal Tc1 cells, suggesting a lack of correlation between the ability of Tc1 cells to synthesize IFNγ and the ability to induce DTH responses.

3) Both Tc1 and Tc2 cells are able to migrate to local inflammatory sites.

D. Discussion.

Despite their distinct Th1- and Th2-like cytokine patterns, Tc1 and Tc2 cells both induced strong footpad swelling, a similar degree of edema and similar neutrophil and macrophage infiltration. Tc2 cells caused significantly more eosinophil infiltration, possibly due to the participation of Tc2 cytokines IL-4 and IL-5. This study demonstrates that DTH-like inflammatory responses can be developed in a Tc2 (Th2) as well as in a Tc1 (Th1) cytokine environment, which also suggests that Th1/Tc1 cytokines are not always essential for DTH, while Th2 cytokines may not always inhibit DTH responses.

IL-4 expresses dual effects on inflammatory responses. Negative effects include the inhibition of up-regulation of ICAM-1 and ELAM-1 on endothelial cells (Thornhill et al., 1990), and suppression of the thrombomodulin pathway (Kapiotis et al., 1991), which is normally involved in inflammation. However, in synergy with TNF or IL-1, IL-4 enhances the expression of VCAM-1 on endothelial cells both in vitro (Masinovsky et al., 1990) and in vivo (Briscoe et al., 1992), which correlates with increased adhesion and probably infiltration of T cells and eosinophils (Thornhill et al., 1991; Schleimer et al., 1992). In the presence of TNF or IFNγ, IL-4 also induces morphological changes of the endothelial cells and increases vascular permeability (Klein et al., 1993). These aspects suggest that IL-4 can play either a positive or a negative role during inflammation, depending on other environmental factors. In the Tc2-injected footpads, both IL-4 and TNF were produced, therefore these two cytokines in synergy could induce both edema and cellular infiltration.

IL-10 inhibited both tissue edema (Li et al., 1994) and cellular infiltration (Powrie et al., 1993) during the effector stage of DTH induced by Th1 clones and *Leishmania* antigens, respectively. But large amounts (e.g. 20-100μg/mouse) of IL-10 were required for the DTH inhibition. Although Tc2 and to a lesser extent Tc1 cells produce IL-10 in response to Con A in vitro, no IL-10 was detected (or less than 0.1ng/foot) in either Tc1 or Tc2 injected footpads, suggesting that the in vivo production of IL-10 may be too low to inhibit DTH. Alternatively, IL10 may be used up rapidly in the footpad environment, so that effective concentrations remain low. Even if IL-10 was produced during Tc cell induced DTH, it could be a positive mediator to the inflammation, as IL-10 can chemoattract CD8 T cells (Jinquan et al., 1993), and transgenic expression of IL-10 in the islets of Langerhans led to a pronounced cellular infiltration in the pancreas (Wogensen et al., 1993).

The similar DTH reaction induced by Tc1 and Tc2 cells indicated that this response may be regulated by common products of Tc1 and Tc2 cells, such as TNF. TNF is a potent activator of vascular endothelial cells, which are key players in local inflammatory responses. TNF not only increases production of cytokines (e.g. IL-6) and chemokines (e.g. IL-8) by endothelial cells, but also upregulates the expression of molecules involved in leukocyte adhesion (e.g. ELAM-1, ICAM-1 and VCAM-1) (Pober and Cotran, 1990), thereby inducing granulocyte and monocyte infiltration. Furthermore TNF induces morphological alterations of the endothelial cells, increased vascular leakage and tissue edema. TNF can also be produced by infiltrated macrophages and neutrophils, thus secondary TNF could further contribute to the inflammation. TNF has been reported as a common mediator for DTH induced by both Th1 and Th2 cells (Muller et al., 1995b). Chemokines, which are important in cell adhesion and extravasation, could also be potential mediators of DTH responses induced by Tc1 or Tc2 cells. Although Th1, Th2 and Tc1 cells all produce chemokines (Cherwinski et al., 1987; Fong and Mosmann, 1990), production by Tc2 cells is not yet known.

Th2 and Tc2 cells produce similar cytokines, but Tc2 cells are much more active in mediating adoptively-transferred DTH responses, which suggested that other than the cytokine pattern, there were additional factors that could cause the difference in DTH induction by these cells. Although resting Th2 cells fail to induce footpad DTH, preactivated Th2 cells induced comparable levels of footpad swelling to that induced by Th1 cells. The requirement for preactivation in vitro suggests that Th2 cells cannot be fully activated in vivo. It is possible that Th2 may need more costimulators than Th1 cells, or different types of APCs. CD8 T cells could contact more allo-APCs in vivo, as there are probably more MHC class I positive cells than Class II positive cells in the footpads. Both Tc1 and Tc2 cells induced a peak reaction (16-20 hr) slightly earlier than that induced by Th1 cells (24 hr). The detection of T cell cytokine production at early time points, and the similar DTH reaction induced by resting and activated Tc1 or Tc2 cells, also suggested a rapid activation of these cells in vivo.

The production of IFNγ often correlates with DTH. However, similar magnitude of footpad swelling were induced by normal Tc1 cells; cytokine deficient Tc1 cells, which produced much less IFNγ both in vitro and in vivo; and Tc2 cells, which did not produce detectable levels of IFNγ. Furthermore, anti-IFNγ Ab did not inhibit the tissue swelling of Tc1 cell-induced DTH. These results suggested either the amount of IFNγ needed to induced the responses was low, or with the presence of other factors, IFNγ was not absolutely necessary for the DTH induced by CD8 cells. The later hypothesis was supported by the findings that DTH to herpes simplex viral Ag was induced similarly in IFNγ deficient mice to that in the control mice (Bouley et al., 1995).

Neither Tc1 nor Tc2 cells (10^7 cell/mouse) were efficient in inducing footpad DTH when transferred systematically into syngeneic mice. In the inflamed footpads in which cold Tc cells were injected with the allo-APCs (Figure 5.30), the numbers of the migrated radio-labeled Tc cells were calculated by their radioactivity. Among the injected 10^7 Tc cells, only less than 10^5 cells were recruited in the inflamed feet. The cell number could be even less in the APC challenged footpads when testing the systemically transferred DTH. As at least $2x10^5$ cells were required for inducing a significant footpad swelling when transferred locally (Figure 5.3), it was not so suppressing that the systemically transferred Tc cells did not induce a strong DTH. Furthermore the low efficiency of local migration of Tc cells could be due to a poor initiation of inflammation by the APC challenge. During the initiating stage of DTH, the DTH-initiating cells were activated after antigen challenge. These cells then release an antigen-specific DTH initiating factor that activated mast cells to produce serotonin. Local release of serotonin opened the gaps of endothelial cells of the blood vessels, vasodilated and perhaps increased the expression of adhesion molecules on the endothelial cells, which led the recruitment of circulating T cells (Askenase, 1992). The challenge cells used in this study were either B lymphoma or a macrophage cell line, which may not be able to induce local serotonin release, therefore may not be able to initiate a DTH response efficiently.

Even in locally transferred DTH model, a relatively large number of Tc cells (>2x10^5 cell/mouse) was needed to induce a significant footpad swelling. Analyzed from the footpad cell extracts, it was found that most of the injected Tc cells were in the dead cell population separated by Ficoll, as early as 12 hr after cell injection. The death of most of the injected T cells probably was the reason why a originally large number of cells were required. The cause of the cell death is not clear, it could be due to the lack of growth factor, such as IL-2, or due to the killing of each other through Fas and Fas ligand mediated pathway (see chapter VI).

T cell migration into local inflammatory sites is a critical early step in DTH responses. Although both Tc1 and Tc2 cells were able to preferentially migrate into an inflammatory site, among three experiments, Tc1 showed consistently stronger local migration than Tc2 cells (Figure 5.30, and data not shown). This result indicated that both Tc1 and Tc2 cells have the potential to mediate DTH during immune responses, but Tc1 may be more effective than Tc2 cells.

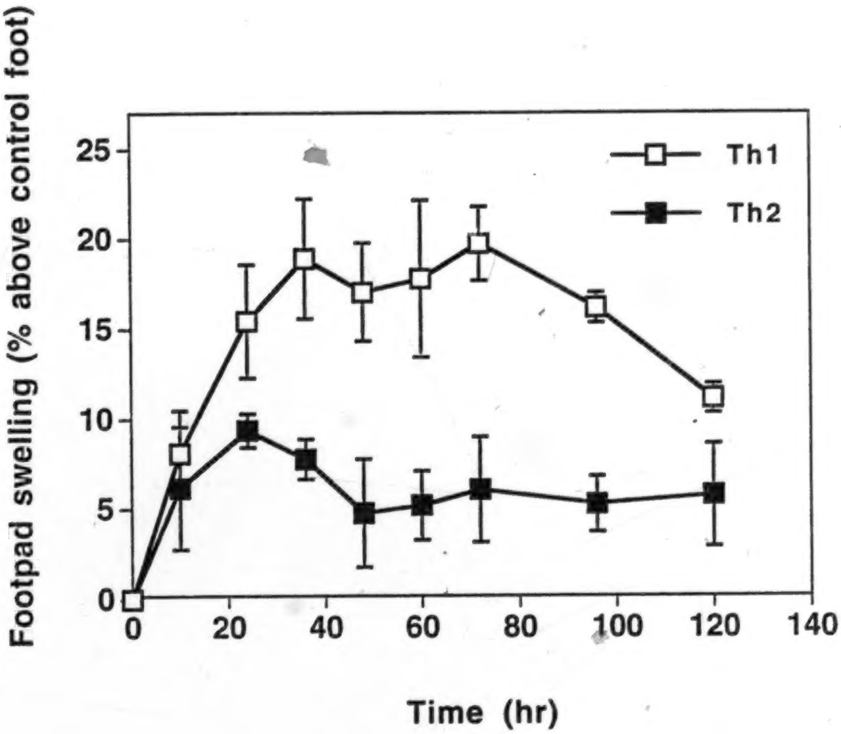


Figure 5.1. Allo-reactive Th1 cells are more active than Th2 cells to induce footpad DTH. Day 10 $H2^b$-anti-$H2^d$ Th1 or Th2 cells (2×10^6 cells/mouse) were injected into the left hind footpads of naive BALB/c mice ($H2^d$). An equal volume of PBS was injected into the right hind footpads of these mice as controls. Footpad swelling was measured at different time points. Each point represents the mean ± SD, (n=3).

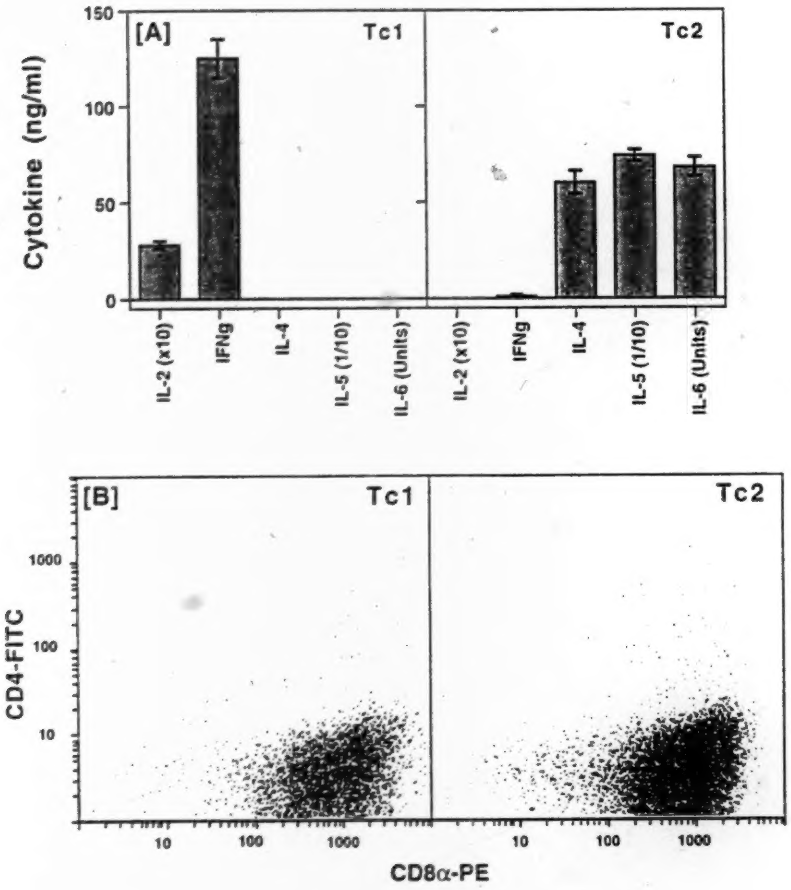


Figure. 5.2. Cytokine profiles and phenotypes of Tc1 and Tc2 cells. CD8$^+$ T cells were purified from naive C57BL/6J (H2^b) splenocytes, and stimulated with alloantigen, M12.4.1 cells (H2^d), in either Tc1- or Tc2-favoring conditions. Seven days after the culture, some of the T cells were restimulated with Con A, and the 24hr Con A stimulated supernatants were tested for cytokines by ELISA (A). Some Tc1 or Tc2 cells were also stained with anti-CD4-FITC and anti-CD8-PE, and their CD4/CD8 expression was detected by FACScan analysis (B).

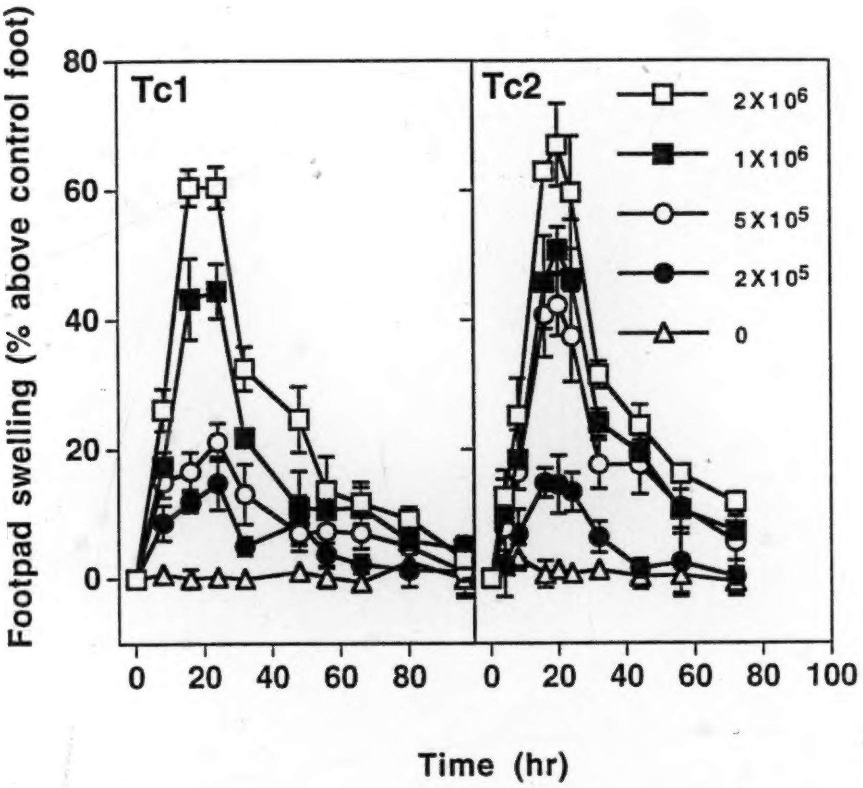


Figure 5.3. Both Tc1 and Tc2 cells induce cell dose-dependent footpad swelling. Different numbers of day 10 $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells were injected into the left hind footpads of naive BALB/c ($H2^d$) mice. The right hind footpads of these mice were injected with an equal volume of RPMI as internal controls. Footpad swelling was measured at different time points for 3-4 days. Each point represents the mean ± SD, (n=5).

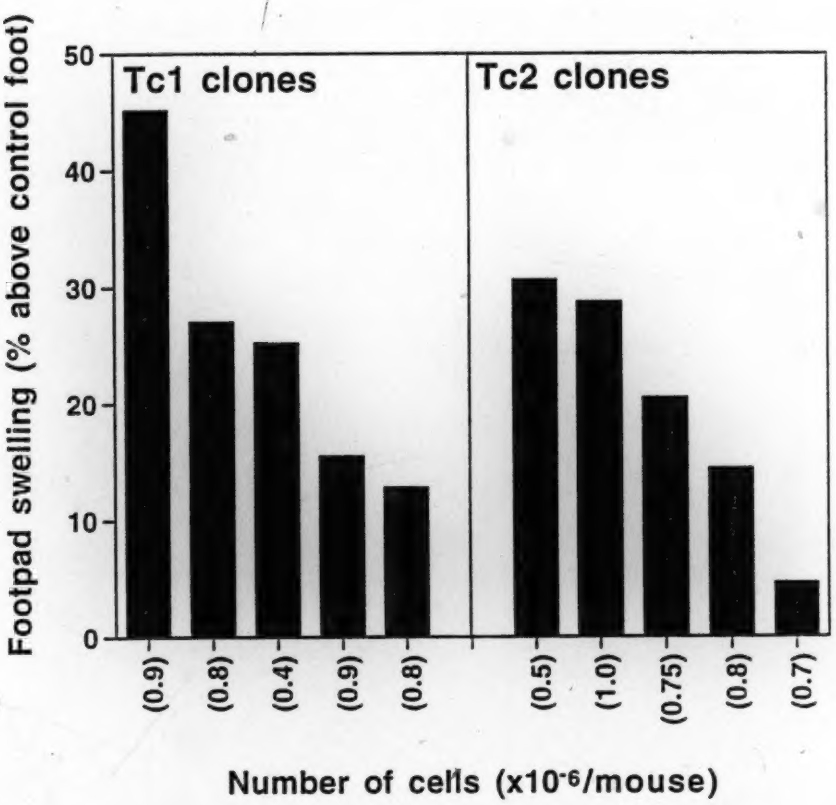


Figure 5.4 Tc1 and Tc2 clones induce footpad swelling. Naive CD8 T cells ($H2^b$) were sorted one cell per well in a 96-well plate by FACS, and the single cells were stimulated with M12.4.1($H2^d$) in either Tc1- or Tc2- culture condition. The clones were restimulated every 7 days until enough cells were obtained for DTH induction. Tc1 or Tc2 cells from each clone were injected into the left hind footpad of one naive BALB/c mouse, and footpad swelling was measured 22 hr after. Each bar represents the footpad swelling of one mouse induced with cells from one Tc clone.

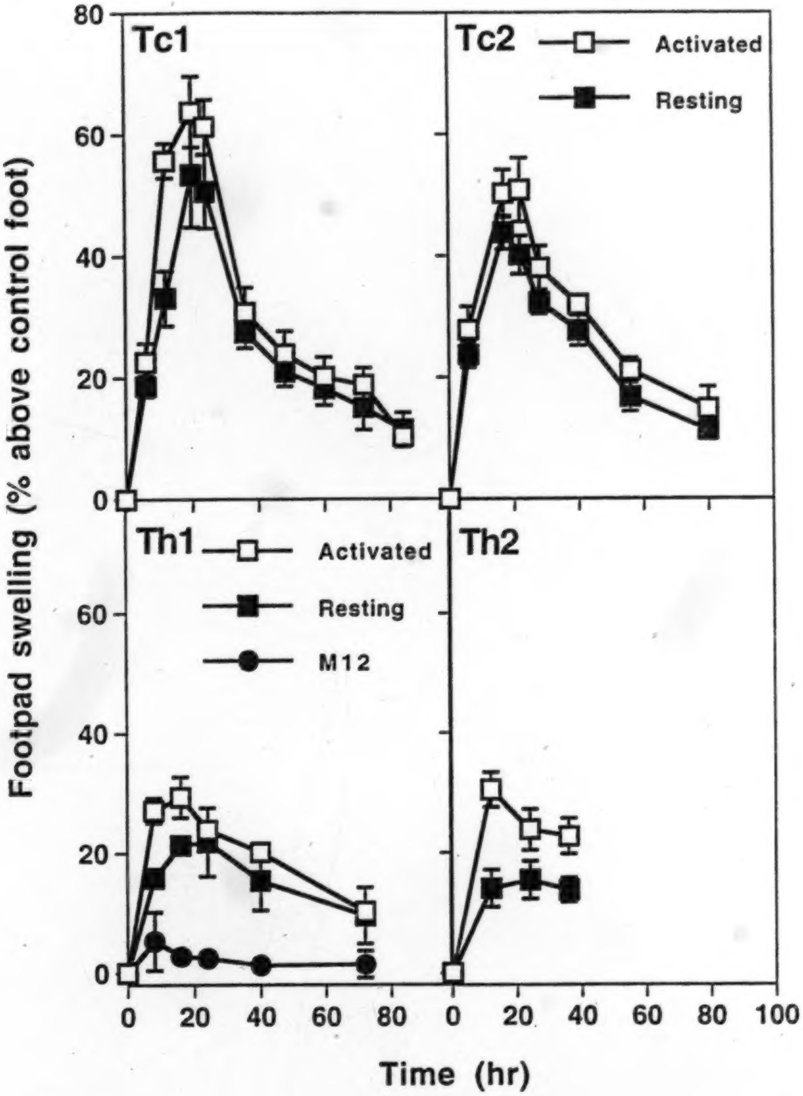


Figure 5.5. Footpad swelling induced by resting or activated Tc1, Tc2, Th1 and Th2 cells. Resting (3 weeks) or activated (restimulated for 24 hr) H2^b-anti-H2^d Tc1, Tc2, Th1 or Th2 cells (10^6/mouse) were injected into the left hind footpads of naive BALB/c mice, the right hind footpads of these mice were injected with an equal volume of RPMI as internal controls. Footpad swelling was measured at different time points. Each point represents the mean ± SD, (Tc1,n=5; Tc2, n=4; Th1and Th2, n=3).

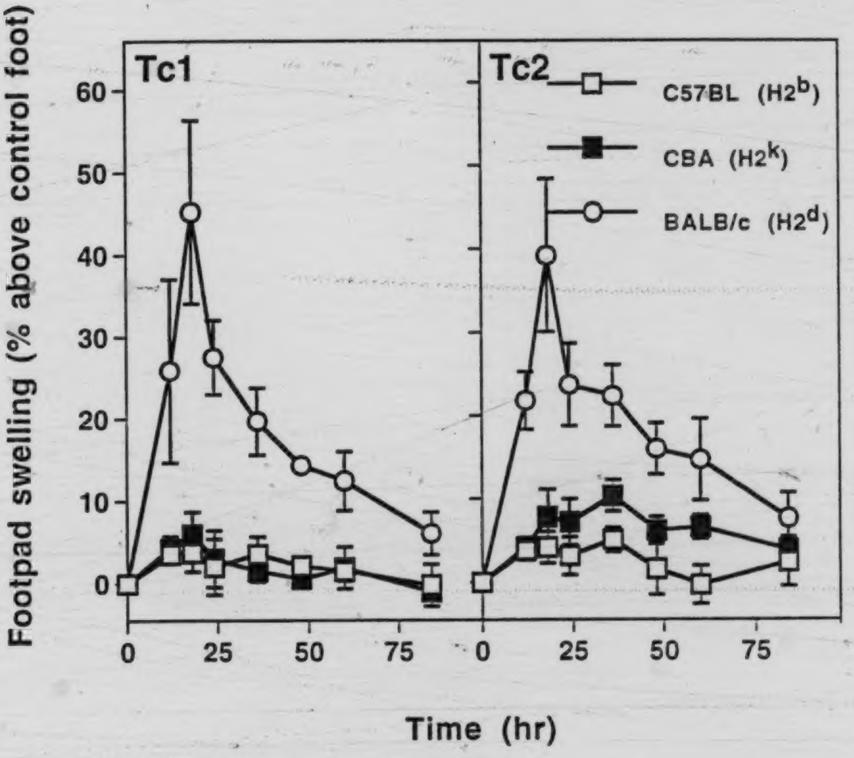


Figure 5.6. Footpad swelling induced by Tc1 or T c2 cells is antigen specific. Day 10 H2^b-anti-H2^d Tc1 or Tc2 cells (10^6 cells/mouse) were injected into the left hind footpads of naive BALB/c (H2^d), C57BL/6J (H2^b) or CBA (H2^k) mice. An equal volume of RPMI was injected into the right hind footpads of these mice as internal controls. Each point represents the mean ± SD, (n=4).

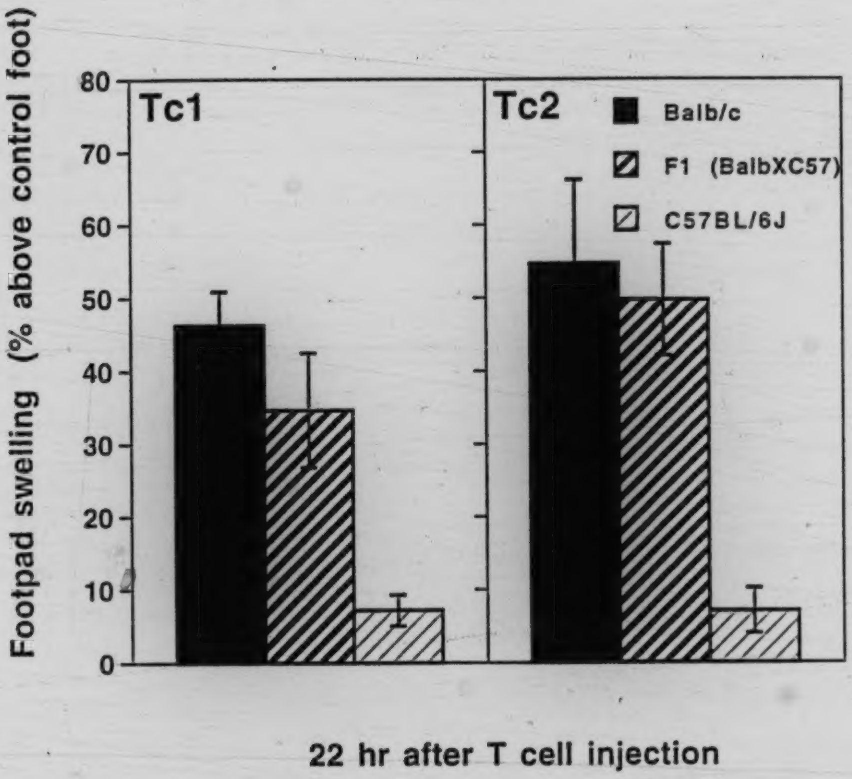


Figure 5.7. Significant footpad swelling is induced in $H2^{d/b}$ as well as in $H2^{d}$ mice by either Tc1 or Tc2 cells ($H2^{b}$-anti-$H2^{d}$). Day 10 Tc1 or Tc2 cells (10^{6}/mouse) were injected into the left hind footpads of naive BALB/c ($H2^{d}$), C57BL/6J ($H2^{b}$) or the F1 of BALB/c x C57BL/6J mice ($H2^{d/b}$), the right hind footpads of these mice were injected with an equal volume of RPMI as internal controls. The footpad swelling was measured at 22 hr after T cell injection. Each bar represents the mean ± SD, (n=5).

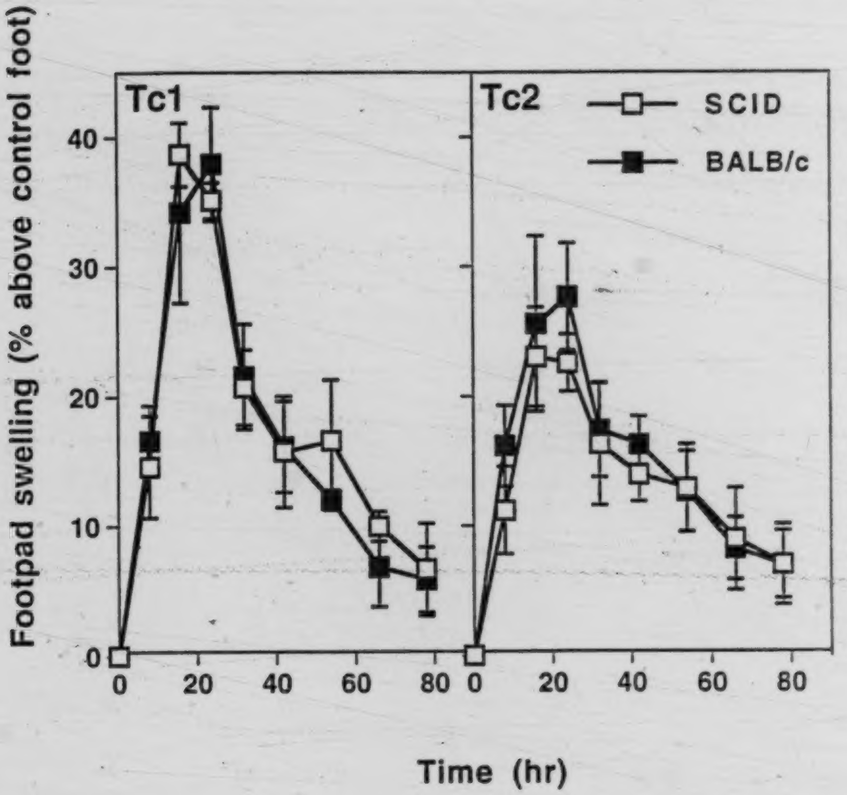


Figure 5.8. Endogenous T cells are not required for the footpad reaction induced by either Tc1 or Tc2 cells. Day 10 $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells (10^6/mouse) were injected into the left hind footpads of SCID ($H2^d$) and BALB/c ($H2^d$) mice, with their right hind footpads receiving an equal volume of RPMI as internal controls. Each bar represents the mean ± SD, (n=5).

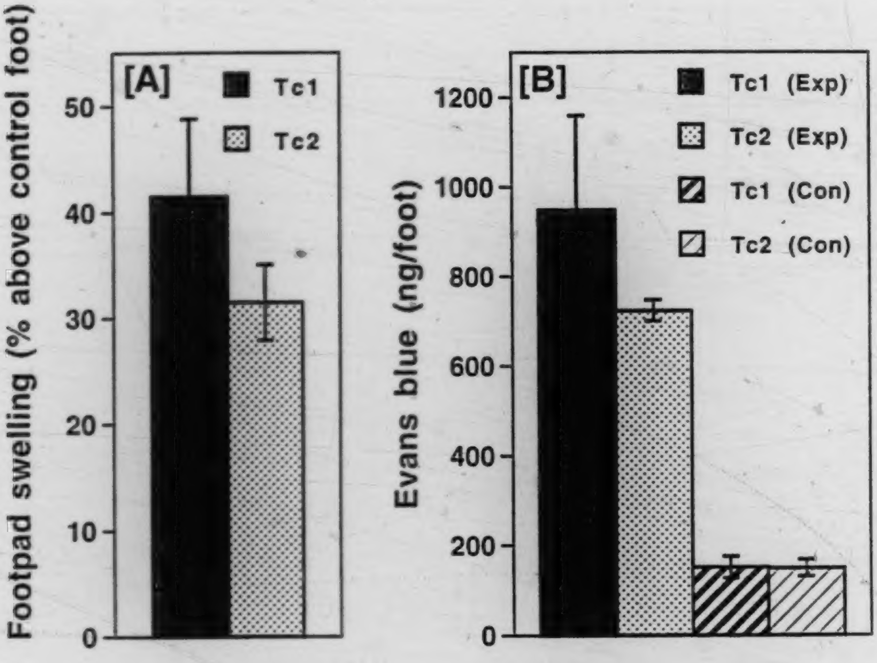


Figure 5.9. Tc1 and Tc2 cells induce similar footpad edema. Day 10 $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells (10^6/mouse) were injected into the left hind footpads of naive BALB/c mice, the right hind footpads of these mice received an equal volume of RPMI as controls. Twelve hr after the T cell injection, 0.1 ml of 1% Evans Blue in PBS was administered iv. Six hr later, footpad swelling was measured, the mice were then sacrificed, perfused, and Evans Blue in each footpad was extracted, and quantitated to represent the vascular leakage. Each bar represents the mean ± SD, (n=4).

Figure 5.10. Similar granulocyte infiltration is detected in the Tc1- and Tc2-injected footpads. Day 10 H2^b-anti-H2^d Tc1 (A, B) or Tc2 (C, D) cells (10^6/mouse) were injected into the left hind footpads of naive BALB/c mice (H2^d) to induce footpad swelling. Twelve hr after T cells injection, the mice were sacrificed and their footpads were frozen and sectioned. The tissue sections were fixed with 4% PFA and stained with anti-Gr-1 antibody (A, C) or the isotype control antibody (B, D). Tissue sections from a Th1 injected footpad (E) and a RPMI injected control footpad (F) were stained with anti-Gr-1 antibody as positive and negative controls, respectively.

Figure 5.11. Similar macrophage infiltration is detected in the Tc1- and Tc2-injected footpads. Day 10 $H2^b$-anti-$H2^d$ Tc1(A, B) or Tc2 (C, D) cells (10^6/mouse) were injected into the left hind footpads of naive BALB/c mice ($H2^d$) to induce footpad swelling. Twenty-four hr after T cells injection, the mice were sacrificed and their footpads were frozen and sectioned. The tissue sections were fixed with acetone and stained with anti-Mac-3 antibody (A, C) or the isotype control antibody (B, D).

Figure 5.12. Anti-Gr-1 staining of cells extracted from Tc1- or Tc2-injected footpads. Footpad DTH was induced by injecting Tc1 (A, B) or Tc2 (D, E) cells into the left hind footpads of BALB/c mice. The right hind footpads of these mice were injected with an equal volume of RPMI as controls (C, F). Footpads were collected 24 hr after T cell injection, cells were extracted and cytospin preparations were made for each individual footpad. Cells were then fixed with PFA and stained with either anti-Gr-1 antibody (A, C, D and F) or the isotype control antibody (B and E).

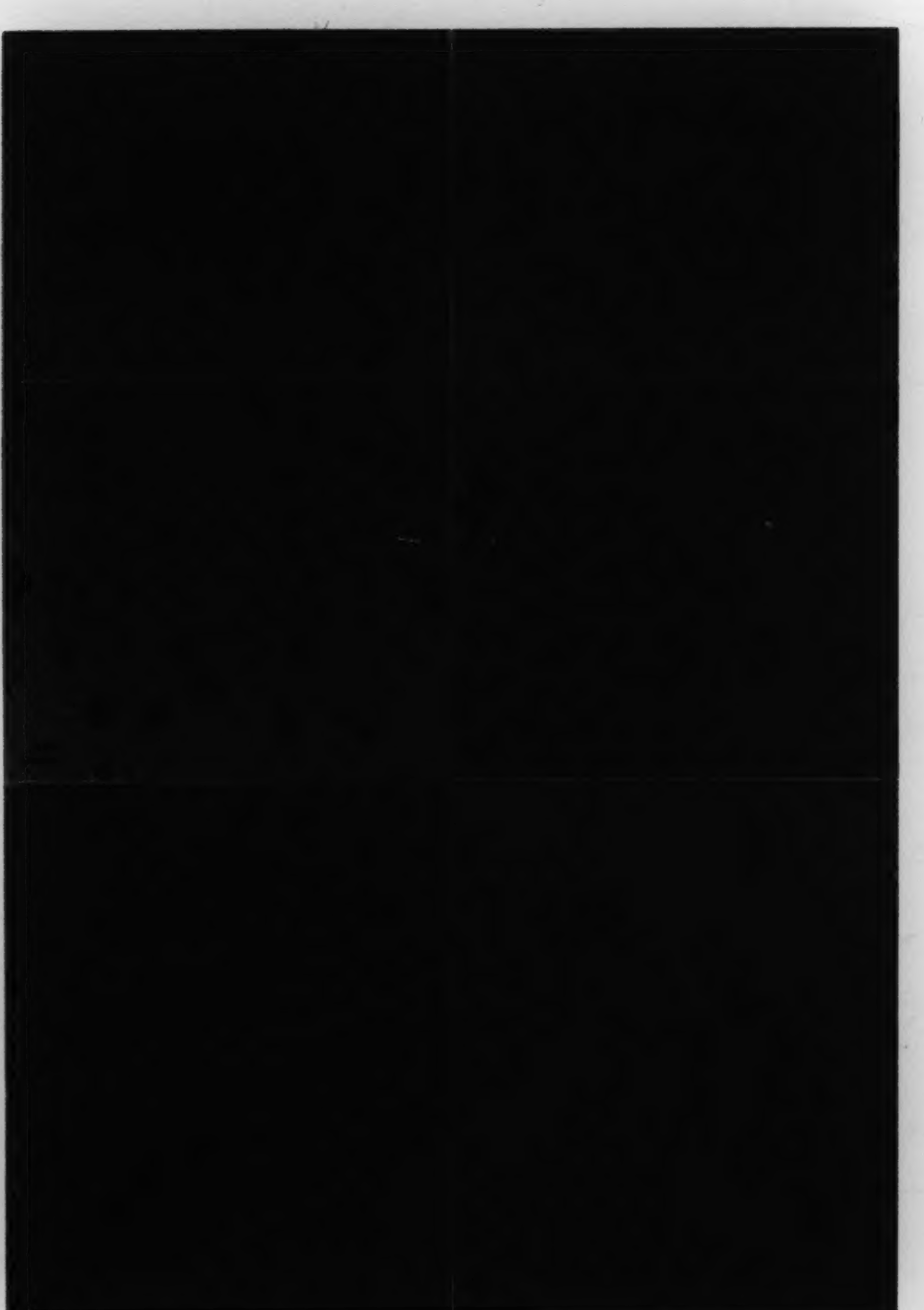
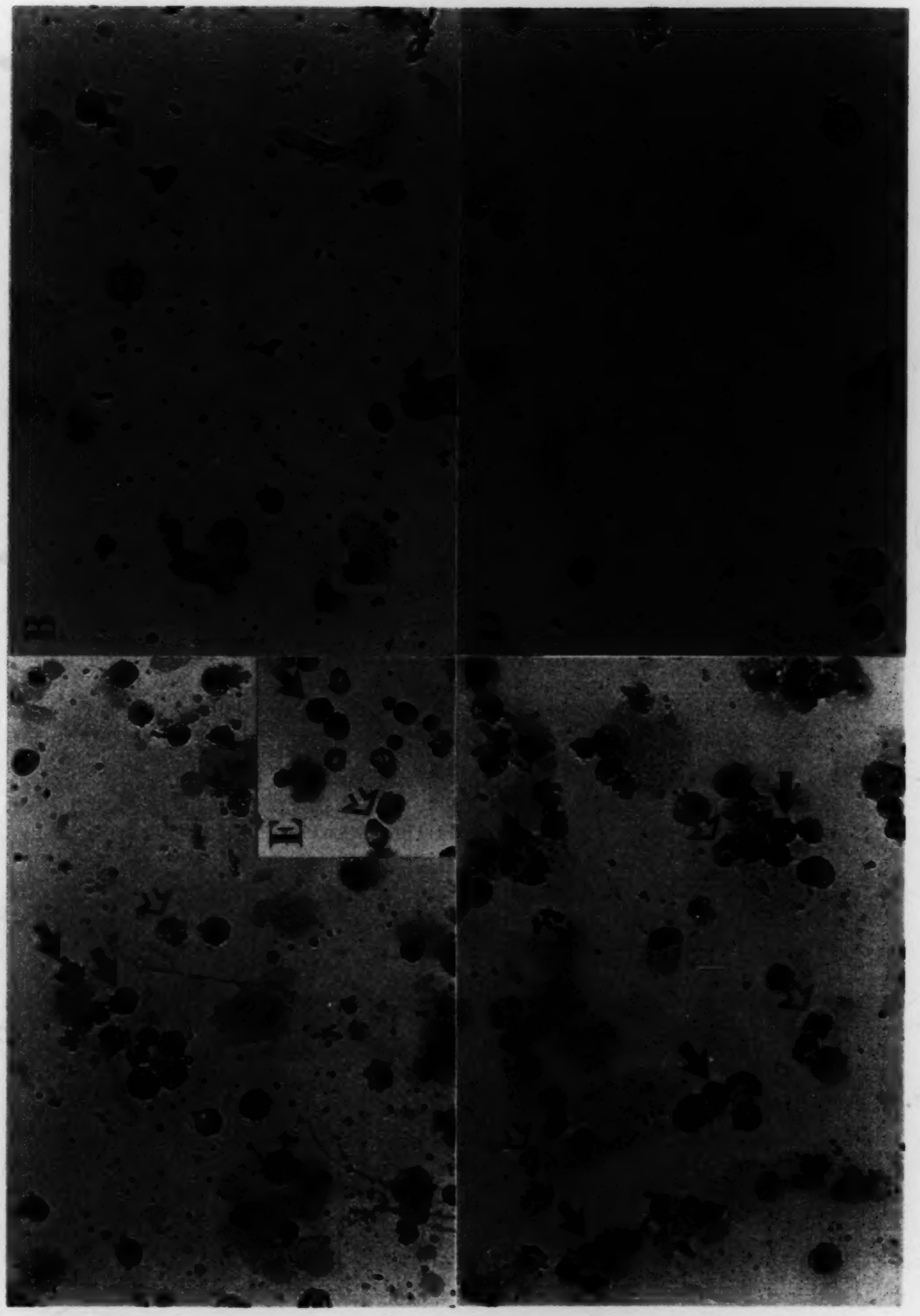
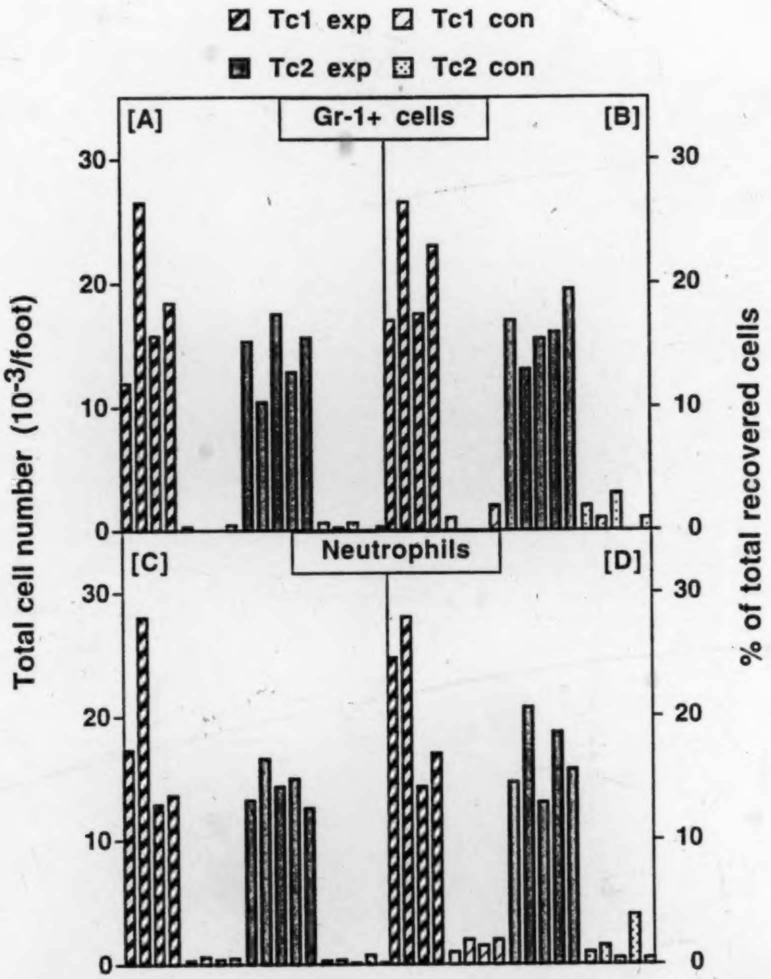


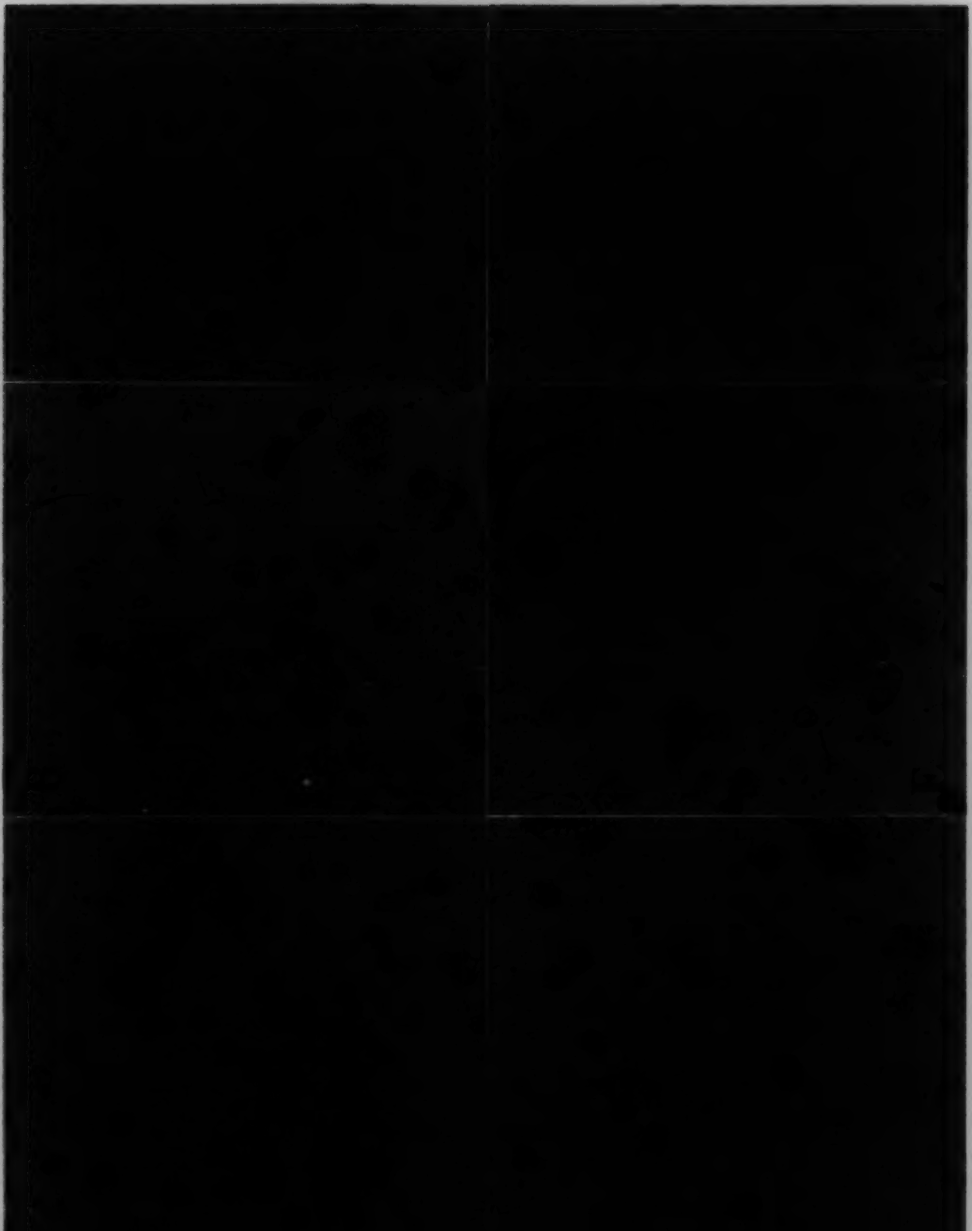
Figure 5.13. Eosin Y and Methylene Blue staining of cells extracted from Tc1- or Tc2-injected footpads. Footpad DTH was induced by injecting Tc1 (A) or Tc2 (C) cells (10^6/mouse) into the left hind footpads of BALB/c mice. The right hind footpads of these mice were injected with an equal volume of RPMI as controls (B, D). Footpads were collected 24 hr after T cell injection, cells were extracted and cytospin preparation was made for each individual footpad. Cells were then fixed with acetone and stained with Eosin Y and Methylene Blue. Peritoneal neutrophils and eosinophils were stained as positive controls (E). For each cytospin preparation, neutrophils (open arrow) and eosinophils (closed arrow) were counted with all the slides coded.

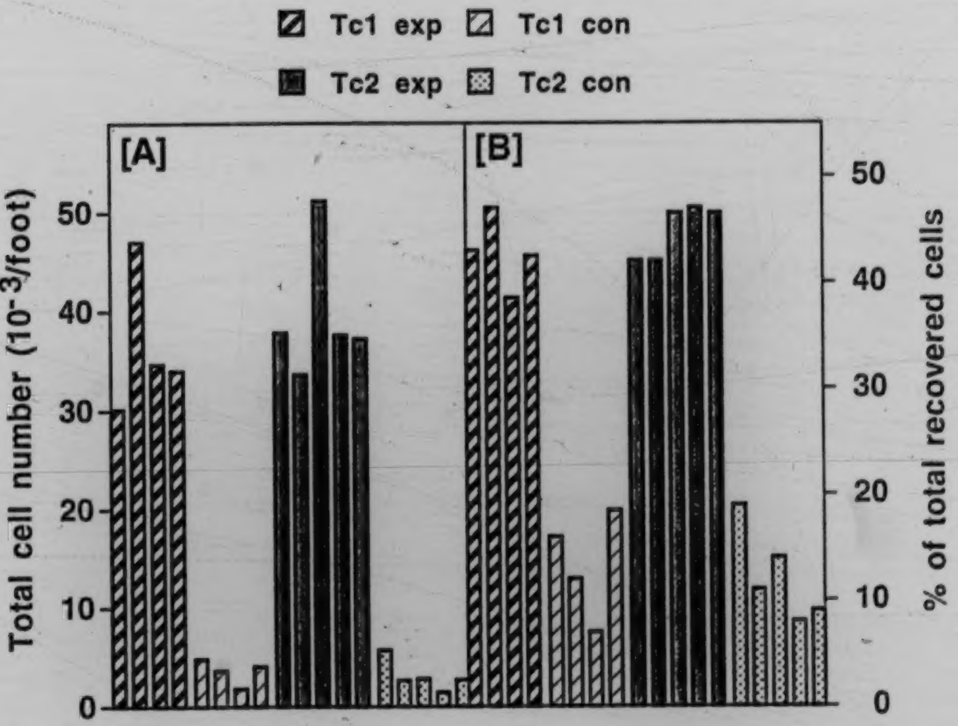

B
E



Figuer 5.14. Similar extent of neutrophil infiltration is detected from Tc1- and Tc2-injected footpads. Footpad swelling was induced by injecting Tc1 or Tc2 cells (10^6/mouse) into the left hind footpads (exp) of BALB/c mice, and the right hind footpads of these mice were injected with an equal volume of RPMI as controls (con). Footpads were collected 24 hr after T cell injection, cells were extracted and cytospin was prepared for each individual footpad. Cells were stained with either anti-Gr-1 antibody (A, B), or with Eosin Y and Methylene Blue (C, D). A total of 200 (Gr-1) or 400 (Eosin Y and Methylene blue) cells were counted for each preparation with all slides coded. The results were shown either by the total cell numbers (A, C) or by the percentage of the recovered cells (B, D). Each bar represents one footpad.

Figure 5.15. Anti-Mac-3 staining of cells extracted from Tc1- or Tc2-injected footpads. Footpad DTH was induced by injecting Tc1 (A, B) or Tc2 (D, E) cells into the left hind footpads of BALB/c mice. The right hind footpads of these mice were injected with an equal volume of RPMI as controls (C, F). Footpads were collected 24 hr after T cell injection, cells were extracted and cytospin preparations were made for each individual footpad. Cells were then fixed with acetone and stained with either anti-Mac-3 antibody (A, C, D and F) or the isotype control antibody (B and E).





Figuer 5.16. Similar extent of macrophage infiltration is detected from Tc1- and Tc2-injected footpads . Footpad DTH was induced by injecting Tc1 or Tc2 cells (10^6/mouse) into the left hind footpads (exp) of BALB/c mice, and the right hind footpads of these mice were injected with an equal volume of RPMI as controls (con). Footpads were collected 24 hr after T cell injection, cells were extracted and cytospin preparations were made for each footpad. Cells were then fixed with acetone and stained with anti-Mac-3 antibody. A total of 200 cells were counted for each preparation with all the slides coded. The results were shown either by the total cell numbers (A) or by the percentage of the recovered cells (B). Each bar represents one footpad.

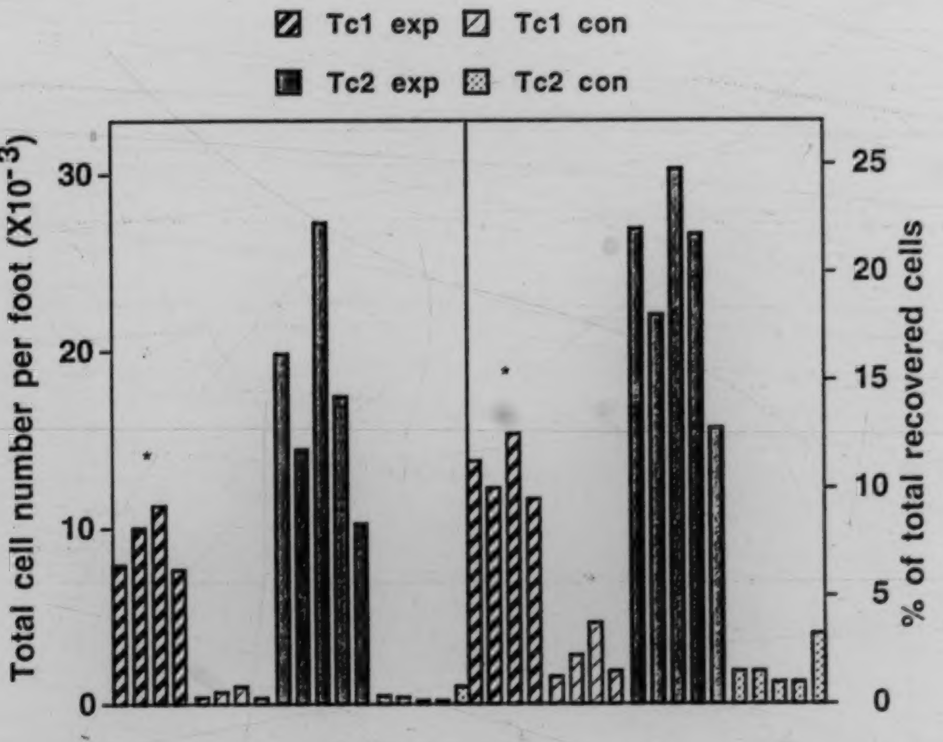


Figure 5.17. More infiltrated eosinophils are detected in Tc2-injected footpads. Footpad DTH was induced by injecting Tc1 or Tc2 cells (10^6/mouse) into the left hind footpads (exp) of the BALB/c mice, and the right hind footpads of these mice were injected with an equal volume of RPMI as controls (con). Footpads were collected 24 hr after T cell injection, cells were extracted and cytospin was prepared for each footpad. Cells were then stained with Eosin Y and Methylene Blue. A total of 400 cells were counted for each cytospin preparation with all the slides coded. Each bar represents one footpad. $^*p<0.05$ eosinophils in Tc1 injected footpads compared to those in Tc2 injected footpads.

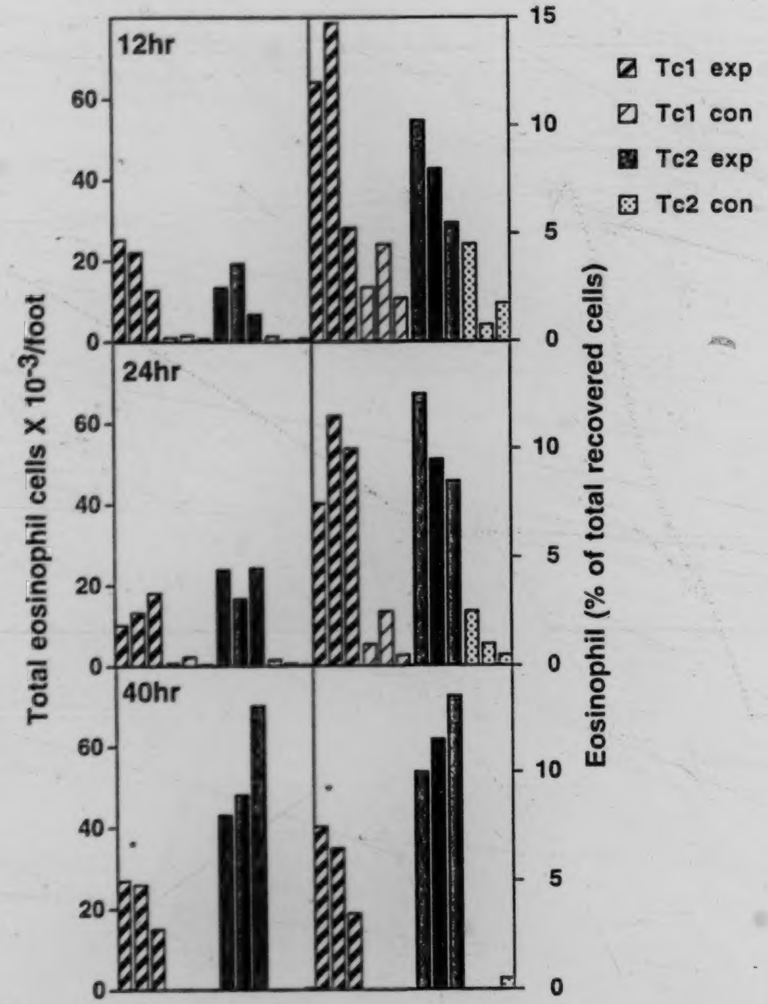
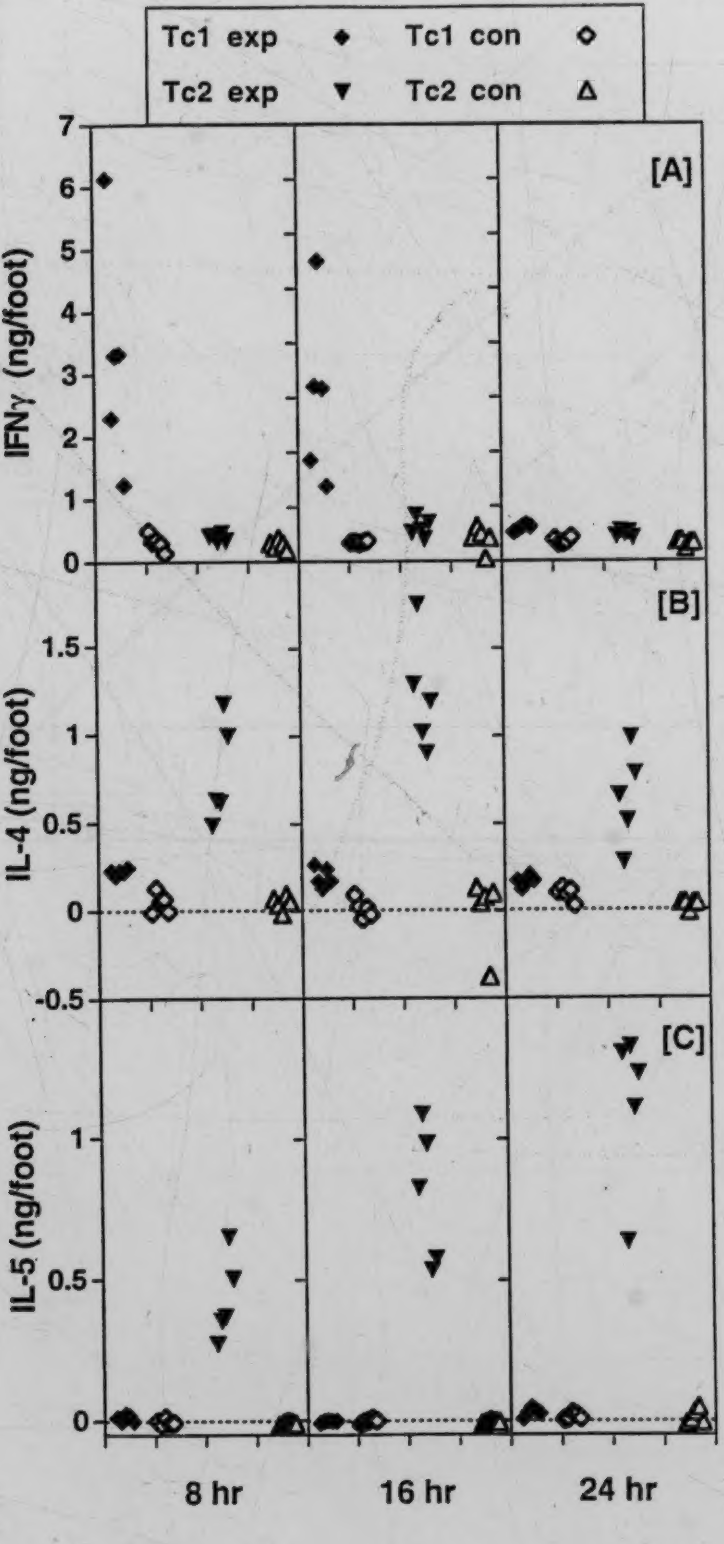


Figure 5.18. Time course of Tc1 or Tc2 cell induced eosinophil infiltration. Footpad swelling was induced by injecting Tc1 or Tc2 cells (10^6/mouse) into the left hind footpads (exp) of BALB/c mice, the right hind footpads of these mice were injected with an equal volume of RPMI as controls (con). Footpads were collected 12, 24, and 40 hr after T cell injection. Cells were extracted and cytospin was prepared from each footpad. Cells were then stained with Eosin Y and Methylene Blue. A total of 200 cells were counted for each cytospin preparation with all the slides coded. Each bar represents one footpad. $^*p<0.05$ eosinophils in Tc1 injected footpads compared to those in Tc2 injected footpads.

Figure 5.19. Tc1 and Tc2 cells retained their in vitro cytokine profiles in the injected footpads. Day 10 $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells (10^6 cells/mouse) were injected into the left hind footpads (exp) of naive BALB/C ($H2^d$) mice, with their right hind footpads received RPMI as controls (con). The mice were terminated at 8, 16, or 24 hr after the DTH induction, and both the experimental and the control footpads were collected. Tissue extract was prepared for each individual footpad, and the levels of IFNγ (A), IL-4 (B) and IL-5 (C) were detected in the tissue extracts by ELISA. Each dot represents one mouse footpad, (n=5).


Tc1 exp ◆
Tc1 con ◇
Tc2 exp ▼
Tc2 con △
[A]
IFNγ (ng/foot)
0
1
2
3
4
5
6
7
[B]
IL-4 (ng/foot)
-0.5
0
0.5
1
1.5
[C]
IL-5 (ng/foot)
0
0.5
1
8 hr
16 hr
24 hr

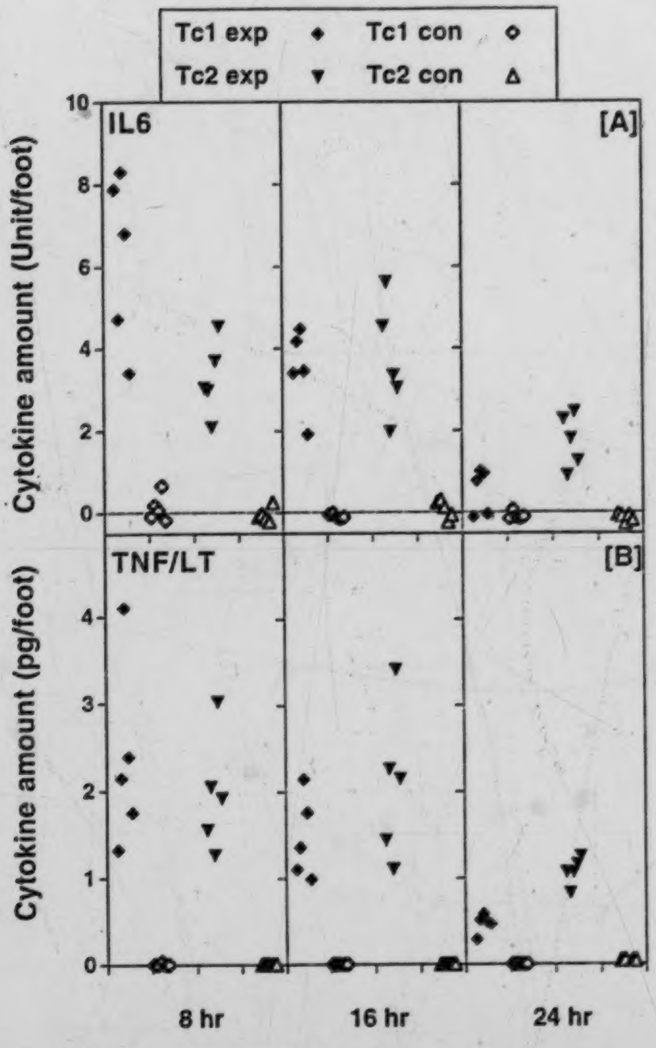


Figure 5.20 Similar levels of inflammatory cytokines are detected in Tc1- and Tc2- injected footpads. Day 10 H2^b-anti-H2^d Tc1 or Tc2 cells (10^6/mouse) were injected into the left hind footpads (exp) of naive BALB/c (H2^d) mice, with the right hind footpads (con) of these mice received RPMI as controls. The mice were terminated at 8, 16, or 24 hr after the DTH induction, and both the experimental and the control footpads were collected. Tissue extract was prepared for each individual footpad, and the level of IL-6 (A) was detected by ELISA, while TNF/LT (B) by a bioassay. Each dot represents one mouse footpad. (n=5)

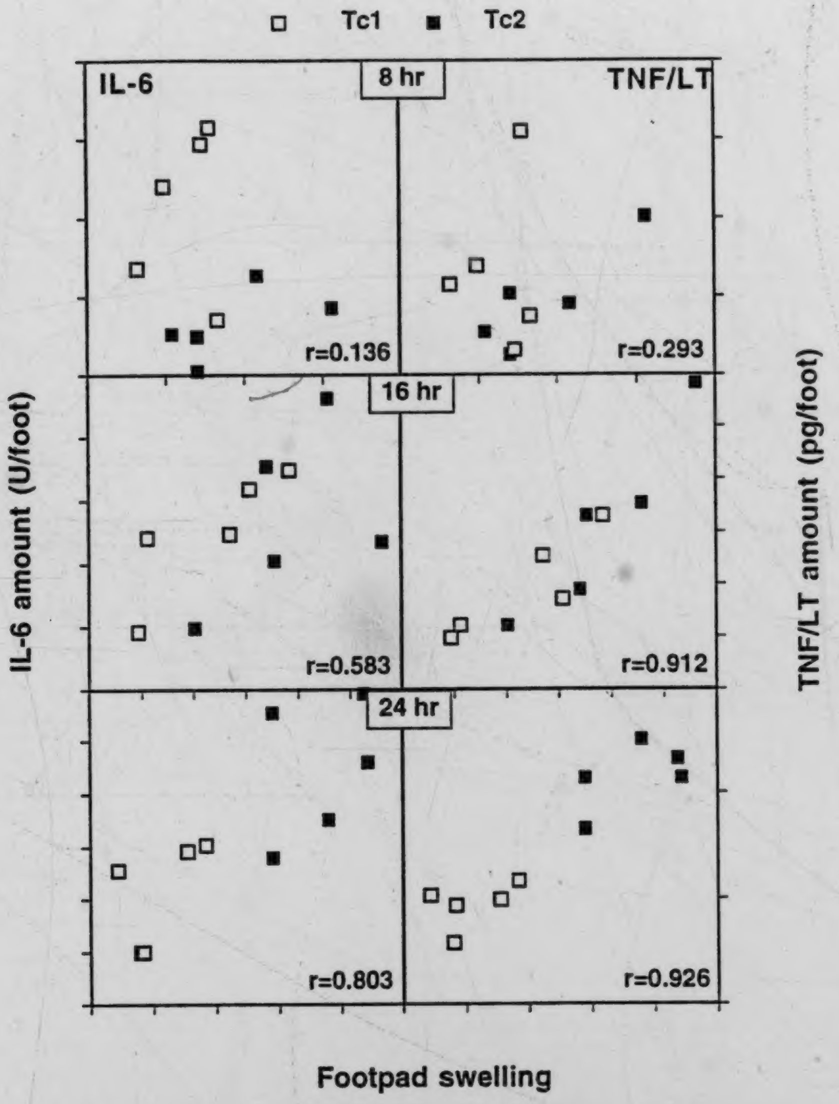


Figure 5.21 Positive correlation between the extent of footpad swelling and the levels of IL-6 or TNF/LT in the corresponding footpads. DTH was induced by injecting Tc1 or Tc2 cells (10^6/mouse) into the left hind footpads of BALB/c mice. At different times after DTH induction, the footpad swelling was measured, and these mice were terminated. Tissue extract was prepared for each individual footpad. The levels of IL-6 and TNF/LT were measured in the footpad extracts by ELISA and a bioassay respectively. The correlation was calculated by a linear curve fit (n=10).

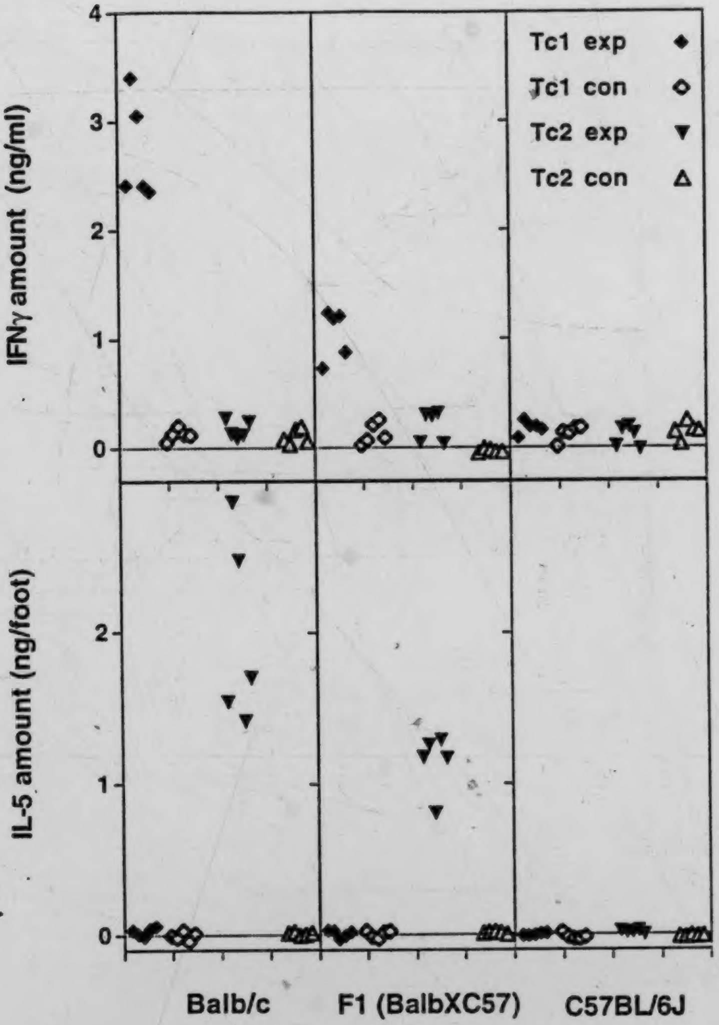


Figure 5.22. Similar Tc1 or Tc2 cytokine profiles are detected in DTH footpads of F1 ($H2^{d/b}$) mice as in BALB/c ($H2^{d}$) mice. $H2^{b}$-anti-$H2^{d}$ Tc1 or Tc2 cells (10^{6}/mouse) were injected into the left hind footpads (exp) of BALB/c, C57BL/6J ($H2^{b}$) and their F1 (BALB/cxC57BL/6J) mice, with their right hind footpads (con) received an equal volume of RPMI as controls. Twenty-two hr after the DTH induction, mice were terminated and tissue extract prepared for each individual footpad. IFNγ and IL-5 were detected in the tissue extracts by ELISA. Each dot represents one mouse footpad. (n=5)

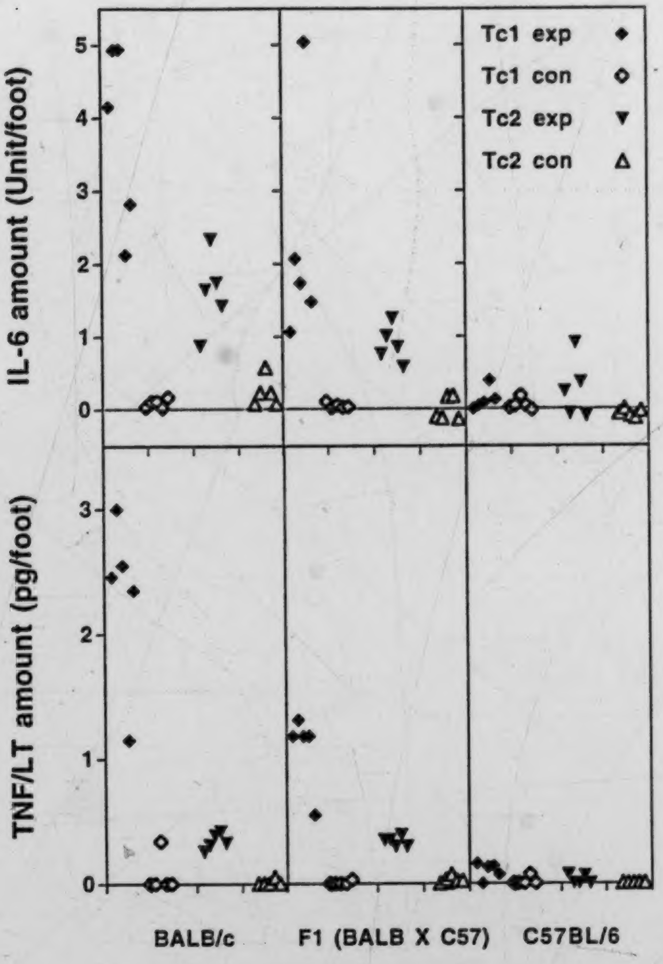


Figure 5.23 Inflammatory cytokines are detected in DTH footpads of F1 ($H2^{d/b}$) as well as BALB/c ($H2^{d}$) mice. $H2^{b}$-anti-$H2^{d}$ Tc1 or Tc2 cells (10^{6}/mouse) were injected into the left hind footpads (exp) of BALB/c, C57BL/6J ($H2^{b}$) and their F1 (BALB/cxC57BL/6J) mice. The right hind footpads of these mice were injected with an equal volume of RPMI as controls (con). Twenty-two hr after the DTH induction, mice were terminated and the tissue extract was prepared for each individual footpad. IL-6 and TNF/LT were detected in the footpad extracts by ELISA and a bioassay respectively. Each dot represents one mouse footpad. (n=5)

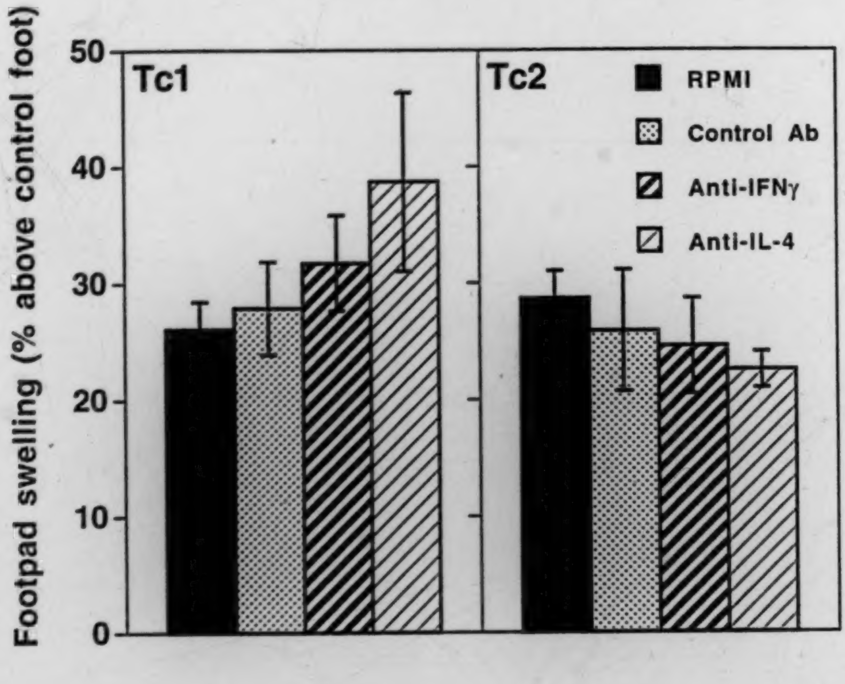


Figure 5.24 Antibody to IL-4 or IFNγ does not inhibit the footpad swelling induced by either Tc1 or Tc2 cells. DTH was induced by injecting $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells (10^6/mouse) into the left hind footpads of naive BALB/c ($H2^d$) mice. The right hind footpads of these mice were injected with an equal volume of RPMI as controls. Antibodies (1mg/mouse) or an equal volume of RPMI were administered to these mice i.p. 1 hr before the DTH induction. Footpad swelling was measured 22 hr after. Each bar represents the mean ± SD, (n=4).

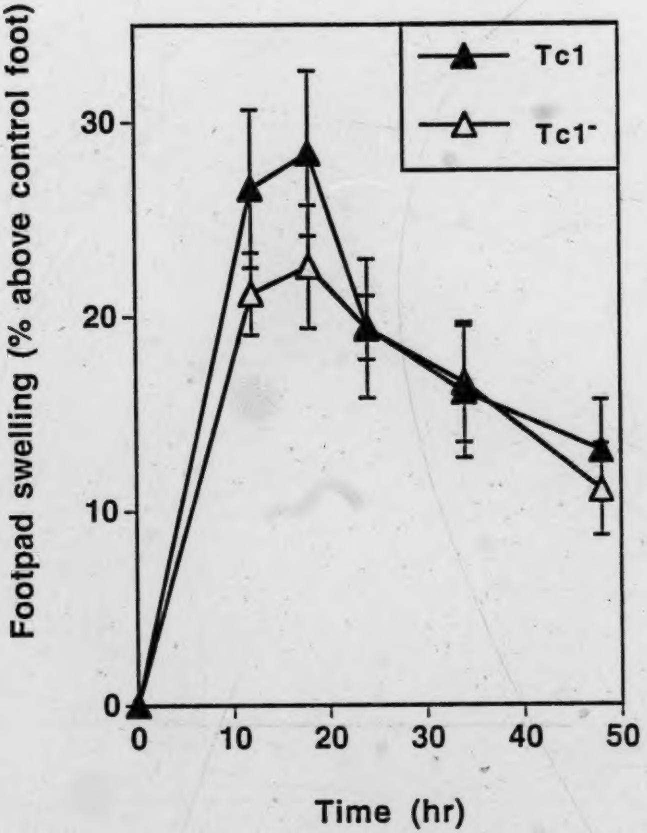


Figure 5.25. Tc1 and the cytokine-deficient Tc1 (Tc1⁻) cells induce similar footpad DTH. H2^b-anti-H2^d Tc1 or Tc1⁻ cells (10^6/mouse) were injected into the left hind footpads of naive BALB/c (H2^d) mice, with their right hind footpads injected an equal volume of RPMI as controls. Each point represents the mean ± SD, (n=3).

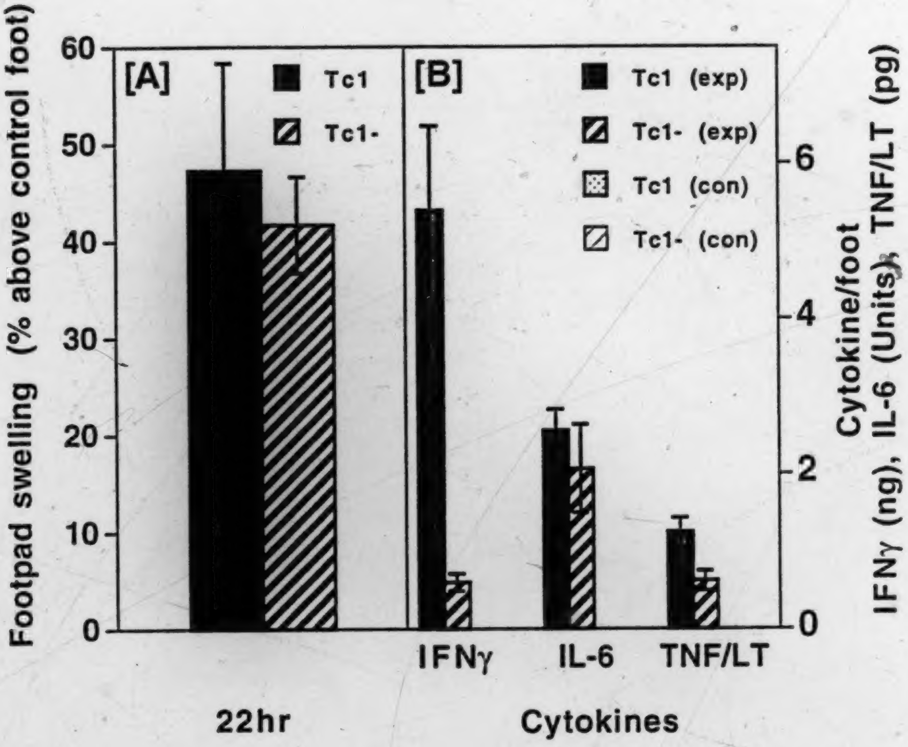


Figure 5.26 Tc1⁻ cells produce less IFNγ in vivo but induce similar footpad DTH as normal Tc1 cells. Footpad DTH was induced by injecting H2^b-anti-H2^d Tc1 or Tc1⁻ cells (10^6/mouse) into the left hind footpads (exp) of naive BALB/c (H2^d) mice. The right hind footpads (con) of these mice were injected with an equal volume of RPMI as controls. Footpad swelling was measured 22 hr after DTH induction. The mice were then terminated, and a tissue extract was prepared for each individual footpad. Cytokines were detected in the footpad extracts by either ELISA (IL-6 and IFNγ) or a bioassay (TNF/LT). Each bar represent the mean ± SD, (n=3).

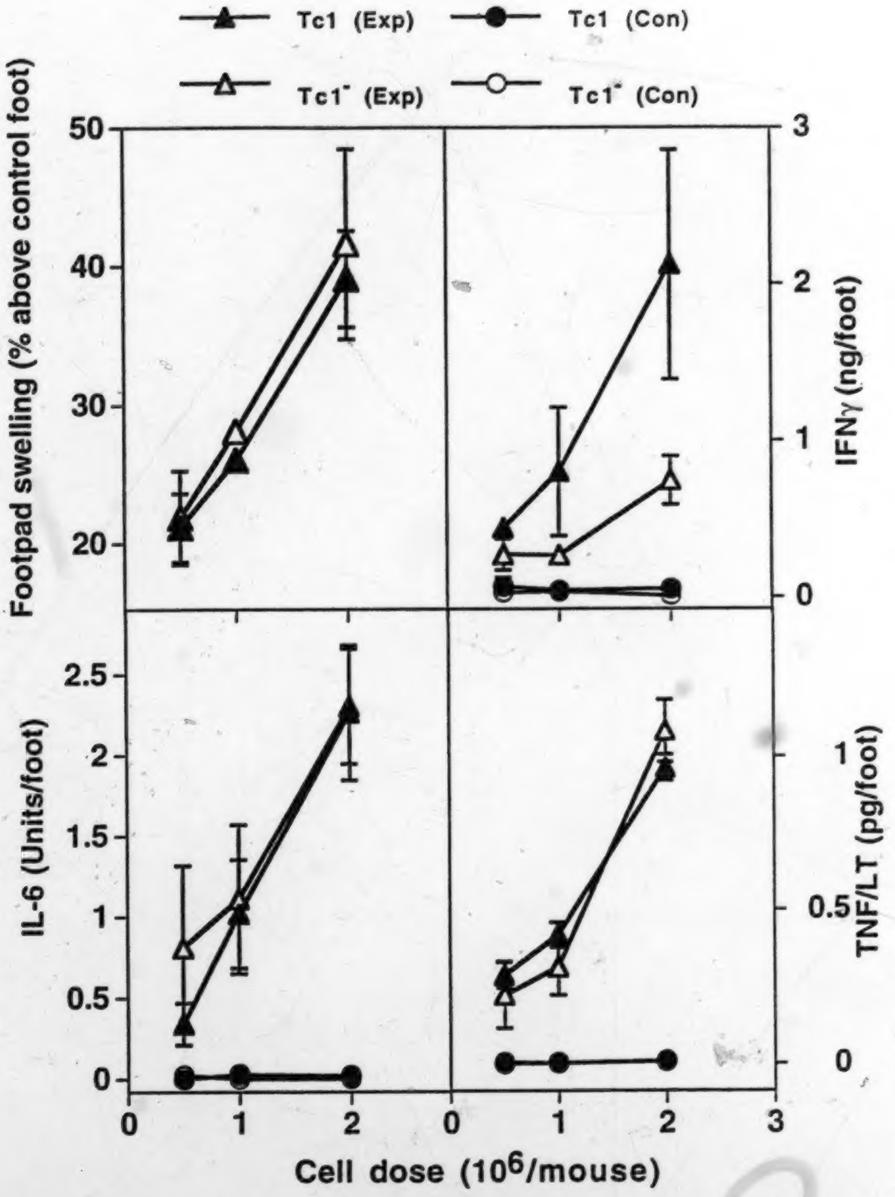


Figure 5.27. Lack of correlation between the in vivo IFNγ levels and the magnitude of footpad swelling induced by Tc1 cells. Footpad DTH was induced by different numbers of Tc1 or cytokine-deficient Tc1 (Tc1-) cells in the left hind footpads (Exp) of naive BALB/c mice, with their right hind footpads receiving RPMI as controls (Con). Footpad swelling was measured 22 hr after T cell injection. Cytokines were detected in footpad extracts by ELISA (IL-6 and IFNγ) or a bioassay (TNF/LT). Each point represents the mean ± SD (n=3).

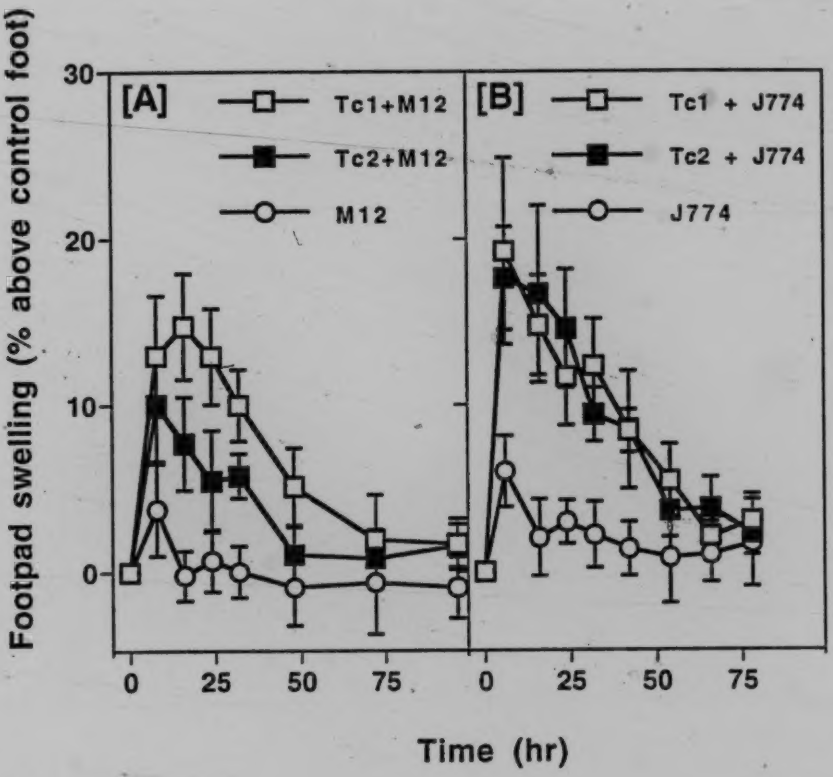


Figure 5.28 Tc1 and Tc2 cells together with target cells induce footpad swelling in syngeneic mice. Day 10 H2^{b}-anti-H2^{d} Tc1 or Tc2 cells (10^{6}/mouse) were injected with either M12.4.1 (A) or J774 (B) (10^{6}/mouse) cells into the left hind footpads of C57BL/6J mice (H2^{b}). The right hind footpads of these mice were injected with 10^{6} Tc1 and Tc2 cells respectively as internal controls. Another group of mice received 10^{6} M12.4.1 or J774 in their left hind footpads, and an equal volume of RPMI in the right hind footpads. Footpad swelling was measured at different time post-cell injection. Each point represents the mean ± SD (n=3).

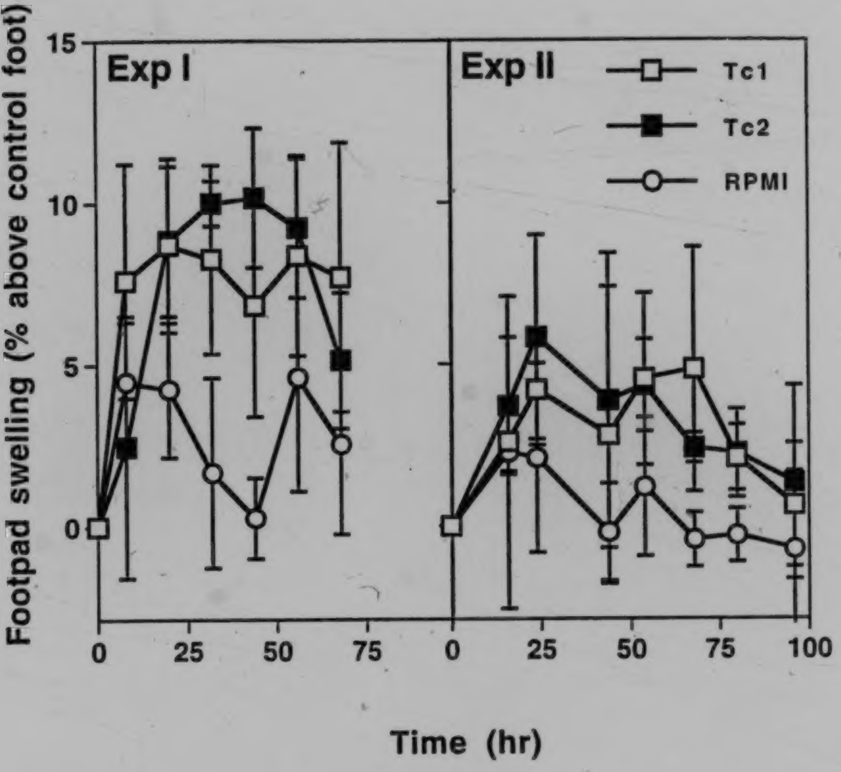


Figure 5.29. Tc1 and Tc2 cells induce marginal footpad swelling when transferred systemically into syngeneic mice. Day 10 H2^b-anti-H2^d Tc1 or Tc2 cells (10^7/mouse) were injected into C57BL/6J (H2^b) mice iv, the control mice received an equal volume of RPMI only. One hr later, these mice were challenged with J774 cells (10^6/mouse) in their left, and an equal volume of RPMI in their right hind footpads. Each point represents the mean ± SD, (n=3).

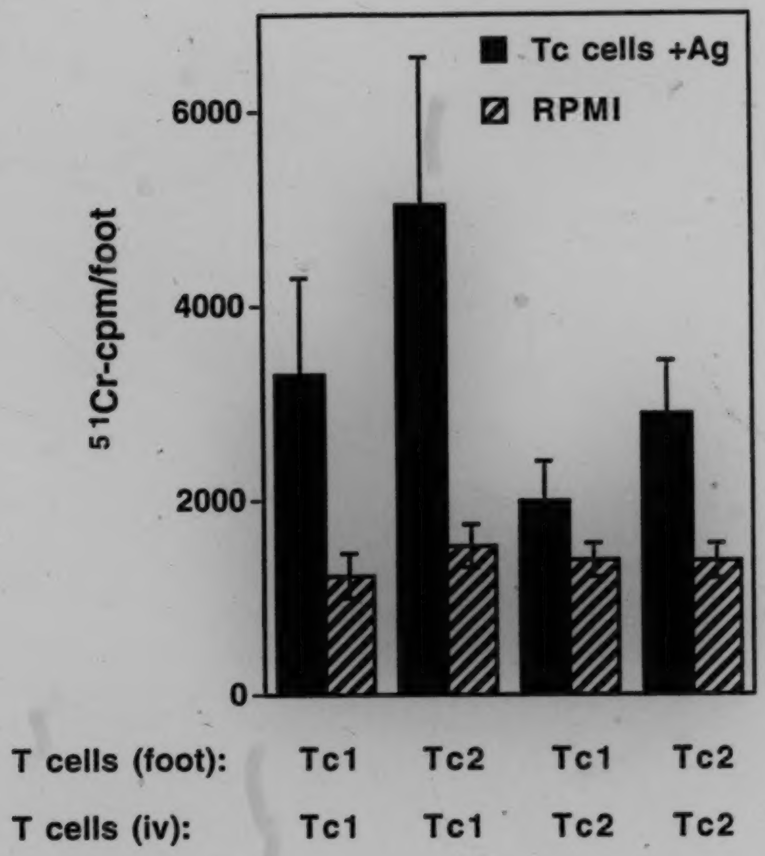


Figure 5.30. Both Tc1 and Tc2 cells preferentially migrate to inflamed sites. $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells ($5x10^5$/mouse) were injected together with $5x10^5$ J774 ($H2^d$) cells into the left hind footpads of C57BL/6J ($H2^b$) mice to induce a local inflammatory response. The right hind footpads of these mice were injected with an equal volume of RPMI as controls. Six hr later, ^{51}Cr labeled Tc1 or Tc2 cells (10^7/ mouse) were injected into these mice iv, another 6 hr later, the mice were terminated and the radioactivity in each foot was measured. Each bar represents the mean ± SD, (n=3).

Table 5.1 A.

Footpad swelling induced by Tc1clones

Clones	Cytokine (ng/ml)*				CD8 expression	Cells injected X10^{-5}	Footpad swelling % above control
	IL-2	IFNγ	IL-4	IL-5			
Tc1							
#1	5.2	18.5	1.2	-	+	8	12.8
#2	0.3	28.5	-	-	+	4.0	25.2
#3	0.3	70.4	-	-	+	9.0	15.5
#4	0.02	52.2	-	-	+	8.0	24.2
#5	0.9	20.6	-	-	+	9.0	40.4

* Assay sensitivity for IL-2 was 30pg/ml; IL-4, 6.5 ng/ml; IL-5, 6.5 ng/ml and IFNγ, 6.5ng/ml

Table 5.1 B.

Footpad swelling induced by Tc2 clones

Clones	Cytokine (ng/ml)				CD8 expression	Cells injected X10^{-5}	Footpad swelling % above control
	IL-2	IFNγ	IL-4	IL-5			
Tc2							
#1	0.02	-	76.9	37.9	+	4.0	21.2
#2	-	-	>1000.0	89.0	+	4.0	20.6
#3	-	-	67.7	41.0	+	3.0	10.8
#4	-	-	588.0	55.3	+	1.0	7.6
#5	-	-	187.9	70.7	+	4.0	25.4
#6	-	-	140.6	89	+	7.5	20.5
#7	-	-	21.4	16.2	+	7.0	4.6
#8	-	-	25.9	164.3	+	10.0	28.7
#9	-	-	27.2	12.9	+	8.0	14.4
#10	-	-	59.3	331.5	+	5.0	19.3
#11	-	-	59.3	500.0	+	5.0	30.6
#12	-	-	44.7	375.6	+	5.0	27.1
#13	-	-	104.7	488.1	+	5.0	16.9

Chapter VI

Effects of perforin-mediated cytotoxicity on DTH induced by CD8 or CD4 T subsets

(Some of the data presented in this chapter have been published in an article entitled "CD8 Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions" by Li Li, Subash Sad, David Kagi and Tim R, Mosmann in *J. Immunol.* (158:4152-4161)

A. Introduction and rationale:

The short-term cytotoxic activity of CD8 T cells is mainly mediated by two distinct mechanisms (Lowin et al., 1994; Henkart, 1994; Kagi et al., 1994b). The first one involves the regulated and polarized secretion of lytic granules, containing perforin and granzymes, upon contact of T cells with target cells (Liu et al., 1995). The second mechanism involves the interaction of membrane-bound Fas ligand on T cells with the Fas molecule on the surface of target cells (Rouvier et al., 1993). In both cases, the killing activities of CD8 T cells induce apoptosis of target cells, which includes condensation of chromatin, blebbing of cell membranes and fragmentation of DNA. The dying cell then shrinks, its endoplasmic reticulum dilates, and finally the cell breaks and forms sealed membrane fragments, called apoptotic bodies (Berke, 1995).

Fas (also named as APO-1 or CD95) is a type-I transmembrane receptor that belongs to the NGF/TNF receptor superfamily, which is characterized by cysteine-rich extracellular domains (Krammer et al., 1994). Fas is expressed on the surface of most hematopoietic cells, and the expression of Fas gene and cell surface protein are enhanced by IFNγ and TNF and by cell activation. In vivo apoptosis via the Fas molecule is triggered by the Fas ligand. The Fas ligand belongs to a TNF-related type-II transmembrane molecule, and is expressed on most killer cells. Killer cells expressing the Fas ligand kill target cells in a Ca^{++}-independent fashion via ligand-Fas interaction (Rouvier et al., 1993). It was proposed that Fas ligand may act as a trimer to induce cell death via the Fas molecule, similar to the action of cell bound TNF. But the apoptosis signal transduced by Fas has not been fully elucidated.

Perforin, the pore-forming molecule, is a complement-like cytolytic mediator carried by both CD8 T cells and natural killer (NK) cells. It is produced and stored within the cytoplasmic granules of these killer cells (Liu et al., 1995). Based on many studies, the potential functions of perforin during target killing was proposed in the following model: Upon conjugation of killer cells with target cells, cytoplasmic granules within the killer cells reorient towards the cell-cell contact site, and contents of the granules, including perforin, are released into the intercellular space. Perforin monomers then bind and insert into the target membrane and polymerize to generate transmembrane pores in the target cell membrane. These perforin pores may assist the delivery of granzymes into target cells. Granzymes are a group of cytoplasmic granule-associated serine proteases (Berke, 1994). In mice, seven granzymes have been identified, named granzyme A-G, and granzyme A and B have been characterized at both gene and protein levels. The delivery of granzymes through perforin pores induces rapid DNA fragmentation and causes apoptosis of the target cells (Berke, 1994). Recently, a substrate of granzyme B has been identified as CPP32, the precursor of a protease responsible for cleavage of poly(ADP-ribose) polymerase (Darmon et al., 1995). This finding provided an explanation for the DNA fragmentation of target cells caused by granzymes. Perforin pores also disturb the membrane integrity of the target cells, and may cause cell death through osmotic lysing (Liu et al., 1995; Berke, 1995).

Perforin is important for the cytotoxic activity of CTLs, but its role in the whole cytolytic function of CD8 T cells was not fully evaluated until the generation of perforin-deficient mice by disruption of perforin gene using homologous recombination (Kagi et al., 1994b; Walsh et al., 1994). These mice are generally healthy with normal lymphoid organs and lymphocytes, and their CTLs appear to contain cytoplasmic granules similar to those of the wild-type cells (Kagi et al., 1994b). However, the cytotoxicity of both T and NK cells against virus-infected or allogeneic target cells was greatly impaired in perforin-deficient mice, and perforin-deficient mice failed to clear noncytopathic lymphocytic choriomeningitis virus (LCMV) (Kagi et al., 1994b). These results demonstrated that perforin is a crucial effector molecule for the cytolytic activities of CD8 T and NK cells. Furthermore, perforin-deficient mice provided a unique possibility to directly test the role of perforin in certain immune responses in which cell-mediated cytotoxicity may be involved.

Allo-reactive Tc1 and Tc2 cells induced strong footpad DTH in mice bearing the target MHC molecules. The potential role of the CTL activity of the Tc cells in the DTH responses is not known. In vitro, both Tc1 and Tc2 cells are cytotoxic to target tumor cell lines as well as to freshly isolated target B cells (Sad et al., 1996). The cytotoxicity of Tc1 and Tc2 cells was mainly through the perforin-mediated pathway, with the Fas pathway contributing to a lesser extent (Carter and Dutton, 1995; Sad et al., 1996). These results implied that Tc1 and Tc2 cells could express perforin-mediated killing to the resident or recruited target cells in the footpads during DTH processes. Since dying cells may release proinflammatory factors, or other danger signals to initiate inflammation after cell damage, the perforin-dependent cytotoxic activity of Tc1 and Tc2 cells could be one of the factors contributing to and enhancing the DTH response. In this project, using Tc cells derived from perforin-deficient mice, the potential contribution of perforin to DTH induced by Tc1 or Tc2 cells was studied.

CD4 cytotoxic T cells have also been documented both in vitro and in certain disease states (Krensky et al., 1982; Golding and Singer, 1985; McCarthy and Singer, 1986; Man et al., 1990). Studies on long-term Th1 clones or short-term nonspecifically activated CD4 splenocytes suggested that different mechanisms might be involved in CD4 and CD8 T cell-mediated cytolysis, and CD4 T cells may not use perforin (Strack et al., 1990; Ju, 1991). However, some Th2 clones expressed perforin-mediated lytic activity like CD8 CTLs (Lancki et al., 1991), and a CD4 bulk population was identified to express perforin- rather than a Fas ligand-dependent cytotoxicity (Williams and Engelhard, 1996). Furthermore, CD4 CTLs using both perforin and Fas have been identified among human cells (Yasukawa et al., 1996, Susskind et al., 1996). Together these results indicated a possible role of perforin in CD4 mediated cytolytic activity. Therefore, perforin-mediated killing was tested on the allo-reactive Th1 and Th2 bulk populations generated in vitro, and the potential effect of perforin on the footpad swelling induced by Th1 and Th2 cells was also studied.

B. Results

Perforin-mediated cytotoxicity enhances but is not critical for DTH induced by Tc1 and Tc2 cells.

To test the potential contribution of perforin-mediated cytotoxicity of Tc1 and Tc2 cells to DTH response, Tc1 and Tc2 cells were generated from perforin-deficient mice ($H2^b$), as well as from normal C57BL/6 ($H2^b$) mice, with allo-stimulation (M12.4.1, $H2^d$). The d 7 perforin-deficient Tc1 and Tc2 cells produced similar cytokines to their normal counterparts, when stimulated with Con A in vitro (Figure 6.1a). Normal Tc1 and Tc2 cells were cytotoxic, but perforin-deficient Tc cells expressed no cytolytic activity towards the Fas-negative target cells in a 4 hr ^{51}Cr release assay (Figure 6.1b).

To study the ability of the perforin-deficient Tc1 and Tc2 cells to induce DTH, d 10 $H2^b$-anti-$H2^d$ Tc1 or Tc2 cells derived from both control and perforin-deficient mice were injected into the footpads of naive BALB/c mice ($H2^d$). In two independent experiments, perforin-deficient Tc1 or Tc2 cells induced either similar or reduced footpad swelling at the peak reaction, compared to those induced by the corresponding control cells (Figure 6.2). These results suggested that perforin-mediated cytotoxicity of the Tc cells was not critical for but may contribute to the magnitude of the DTH responses. The DTH inducibility of perforin-deficient Tc1 and Tc2 cells was further tested using different numbers of T cells. In two independent experiments, both perforin-deficient Tc1 and Tc2 cells induced cell dose dependent footpad swelling, which was again either less than or comparable to that induced by the corresponding control Tc cells (Figure 6.3). Since perforin-deficient Tc cells are able to induce footpad DTH, and in certain experiments, the extent of the footpad swelling induced by perforin-devoid cells was indistinguishable from that induced by control cells, collectively these results suggest that the fast cytolytic activity mediated by perforin may enhance the magnitude but was not crucial for the DTH response induced by either Tc1 or Tc2 cells. The lack of perforin seemed to affect footpad swelling induced by Tc1 cells more than that induced by Tc2 cells, these results were consistent with the generally higher cytotoxicity expressed by Tc1 than Tc2 cells (Figure 6.1b).

To test the in vivo activation of the perforin-deficient Tc1 or Tc2 cells, Tc1 and Tc2 specific cytokines, IFNγ and IL-5, were measured in footpad extracts and compared with those produced by the control Tc cells. In experiments which perforin-deficient and control Tc cells induced comparable footpad swelling, similar levels of Tc1 or Tc2 cytokines were detected in the footpads injected with perforin-deficient or the corresponding control Tc cells (Figure 6.4). These results suggested that perforin-deficient cells were similarly activated as control cells in the footpads. Secondary inflammatory cytokines, IL-6 and TNF/LT were also detected in the tissue extracts from perforin-deficient Tc cell-injected footpads (Figure 6.4). The induction of the inflammatory cytokines by perforin-deficient Tc1 and Tc2 cells again suggested their ability to induce footpad DTH.

Perforin-mediated cytotoxicity does not affect footpad DTH induction by Th1 or Th2 cells.

The contribution of perforin-mediated cytotoxicity to DTH was also tested for CD4 Th1 and Th2 cells. Th1 and Th2 cells were generated from both C57BL/6J ($H2^b$) and the perforin-deficient mice ($H2^b$) with allo-stimulation (M12.4.1, $H2^d$), in the culture conditions of IL-12 with anti-IL-4, and IL4 with anti-IFNγ, respectively. The perforin-deficient Th1 and Th2 cells produced similar cytokines to their normal counterparts, when stimulated with Con A in vitro (Figure 6.5a). In this system normal but not

perforin-deficient Th1 cells often expressed moderate killing to Fas negative target cells in a 4 hr ^{51}Cr releasing assay, while neither normal nor perforin-deficient Th2 cells expressed significant rapid target killing (Figure 6.5b).

To test the potential perforin effects on DTH induction by Th1 or Th2 cells, d 10 H2^{b}-anti-H2^{d} Th1 or Th2 cells, derived from either perforin-deficient or control mice, were injected into the footpads of naive BALB/c mice (H2^{d}). As reported previously, Th1 cells induced footpad DTH with a peak reaction around 24 hr after T cell injection, while Th2 cells caused much lower footpad swelling without a significant peak reaction (Figure 6.6). Perforin-deficient Th1 and Th2 cells showed similar activity to their normal counterparts in DTH induction, in terms of both the magnitudes and time course of the reaction. The effect of perforin on the Th DTH was also tested using different numbers of Th cells. Both normal and the perforin-deficient Th1 cells induced similar dose dependent footpad DTH, while Th2 cells from either normal or the perforin-deficient mice only induced very low (around 10% of control foot) footpad swelling (Figure 6.7). These results confirmed that resting Th1 but not Th2 cells induced footpad DTH, and suggested that perforin did not affect the ability of either Th1 or Th2 cells to adoptively transfer DTH.

The in vivo activation of the normal and perforin-deficient Th1 and Th2 cells was then tested, by measuring cytokine levels in the footpad extracts collected 24 hr after T cell injection. Both normal and perforin-deficient Th1 and Th2 cells retained their specific cytokine profiles in the injected footpads (Figure 6.8). Similar levels of Th1 or Th2 cytokines were detected in the footpads injected with perforin-deficient cells to those in the footpads injected with normal cells (Figure 6.8). These results suggested that the in vivo activation stage of the perforin-deficient cells was similarly to that of the corresponding control cells. In addition, similar levels of secondary inflammatory cytokines, IL-6 and TNF/LT were detected in the extracts of footpads injected with either normal or the perforin-deficient Th1 cells, correlated with the similar footpad DTH induced by these cells (Figure 6.8).

The in vivo cytokine levels were compared between footpads injected with Th1 and Tc1, as well as those received Th2 and Tc2 cells. The in vivo cytokine (IFNγ and LT) levels in the Th1 injected footpads were comparable to those in the Tc1 injected footpads, which correlated with the similar footpad DTH induced by these two T cell subsets (Figure 6.9). But the levels of IL-4 and IL-5 in the Th2 injected footpads were only about one tenth of those in the footpads received Tc2 cells, which also correlated with the lower footpad swelling induced by Th2 cells (Figure 6.9). This result indicated that the Th2 cells may not be equally activated in the footpads as Tc2 cells, which might explain why Th2 cells were not active in DTH induction.

C. Summary of results

1) Perforin-mediated cytotoxicity may enhance but is not critical to the footpad DTH induced by Tc1 or Tc2 cells.

2) Perforin-deficient Tc1 and Tc2 cells produce similar levels of corresponding cytokines to their normal counterparts in vivo, suggesting that these cells are activated similarly in the footpads as normal Tc cells.

2) Normal resting Th1 but not Th2 cells induce footpad DTH, and the lack of perforin has no effect on DTH induction by either Th1 or Th2 cells.

4) Perforin-deficient Th1 cells produce similar levels of cytokines to normal Th1 cells in vivo, suggesting that they can be activated similarly to the control Th1 cells in the footpads. The in vivo cytokine levels produced by Th1 cells are comparable to those produced by Tc1 cells.

5) Both normal and perforin-deficient Th2 cells produce less cytokines than the same numbers of Tc2 cells, suggesting that Th2 cells may not be fully activated in the footpads.

D. Discussion:

CD8 T cells can mediate DTH or other inflammatory responses in many diseases. Since CD8 T cells are cytotoxic against target cells, it has always been a question whether the direct killing of certain target cells by CD8 CTLs contributes to the inflammatory responses mediated by these T cells as a result of the dying target cells. Tc1 and Tc2 cells induce strong footpad DTH, and both subsets are highly cytotoxic mainly through the perforin pathway, therefore, the perforin-deficient Tc1 and Tc2 cells provided an unique system to test the potential contribution of perforin-mediated killing to DTH reactions. Despite the lack of rapid cytolytic activity in a 4 hr ^{51}Cr releasing assay, the allo-specific Tc1 and Tc2 cells derived from perforin-deficient mice were able to induce footpad DTH in mice bearing the target MHC, although in certain cases the magnitude of swelling induced by perforin-devoid cells (especially Tc1) was significantly less than those induced by the control cells. These results suggested that perforin was not critical for Tc cells to induce DTH, but it might enhance the reaction by mechanisms which need to be elucidated.

Tc1 and Tc2 cells used in this study were anti-H2^{d} allo-reactive, therefore theoretically most cells in the footpads of BALB/c (H2^{d}) mice were potential targets for the injected Tc cells. Thus, the allo-system would allow the perforin-mediated cytotoxicity of Tc1 and Tc2 cells to express its maximal effect on DTH reactions. However even in the full allo-reactive system, the direct perforin-mediated target killing by CD8 T cells was not crucial for DTH responses. Therefore, perforin may show even less influence on DTH in other systems, such as virus infection, in which only fewer infected cells can stimulate Ag-specific CD8 T cells.

The non-crucial effect of perforin on DTH responses does not rule out the potential contribution of general target killing to DTH, because besides the perforin-mediated cytotoxic pathway, Fas also plays an important role in short-term target killing. In vitro assays, substantial killing activity against certain target cells still remained in perforin-devoid CTLs, and this perforin-independent killing activity was not revealed if Fas negative target cells were used (Lowin et al., 1994; Henkart, 1994; Kagi et al., 1994b), suggesting that perforin- and Fas-based cytolytic pathways accounted for almost all the T cell-mediated short-term cytotoxicity in vitro. Fas is expressed on most hematopoietic cells, which are the potential targets for Fas-mediated killing in vivo. The effect of Fas-mediated target killing on DTH can be tested in lpr mice, in which the Fas gene is mutated. The effect of total rapid killing on DTH can be studied using perforin-deficient Tc1 or Tc2 cells to induce DTH in lpr mice. Furthermore, as the DTH process lasts for days, which is beyond the time course of rapid killing, TNF-mediated cytotoxicity could also play a role in the DTH responses.

The role of perforin or perforin-mediated cytotoxicity was studied by other groups in several intracellular infectious disease models. Perforin-deficient mice failed to clear noncytopathic LCMV (Kagi et al., 1994a), despite the existence of corresponding antigen-specific CD8 T cells in vivo. However the lack of perforin did not affect the clearance of cytopathic viruses, such as Vaccinia, vesicular stomatitis virus (VSV) and Semiliki forest virus (SFV) in the infected mice (Kagi et al., 1995). These results demonstrated that perforin-dependent cytotoxicity mediated by T cells was crucial for protection against noncytopathic viruses, whereas infections with cytopathic viruses may be controlled by other mechanisms. Mice depleted of perforin also failed to develop a protective reaction against a secondary listeria, a intracellular bacterium, infection (Kagi et al., 1994c).

Cytotoxic T cells are believed to be one of the major cell types that mediate acute allograft rejection. Although perforin-deficient CTLs did not exhibit allo-specific cytotoxic activity in vitro, fully allogeneic hearts were rejected with a similar rate in the perforin-deficient mice and the control mice, with the graft infiltrated predominately by CD8 T cells (Schulz et al., 1995). In another report, perforin-deficient and control mice showed equally full rejection of Fas-negative and TNF-insensitive allogeneic tumor cells (Walsh et al., 1996), although CD8 T cells were the crucial players in the rejection. Furthermore similar rejection of allo-pancreatic islets was also observed in perforin-devoid and control mice (Lu et al., 1995). These results together suggested that although CD8 T cells were crucially involved in graft rejection, this immune response was not correlated with the perforin-mediated cytotoxicity of the allo-reactive CD8 T cells.

The role of perforin was also studied in a diabetic model. In transgenic mice expressing the glycoprotein of LCMV (LCMV GP) in pancreatic beta cells, LCMV infection leads to a potent LCMV GP specific T cell response resulting in rapid development of diabetes. However in the perforin-deficient LCMV GP transgenic mice, insulitis occurred without the development of diabetes (Kagi et al., 1996). In addition, adoptive transferring of activated LCMV-GP-specific TCR transgenic and perforin-deficient T cells led to marked insulitis without diabetes in the LCMV GP transgenic mice. These results indicated that although perforin is required for destruction of beta islet cells, it is not critical for initiating an inflammatory response in pancreatic islets.

Collectively, the above results demonstrated the potential role of perforin in different immune responses mediated by CD8 T cells. Although perforin-mediated cytotoxicity is a major function of CD8 T cells, it is not absolutely required for inflammatory responses induced by CD8 T cells.

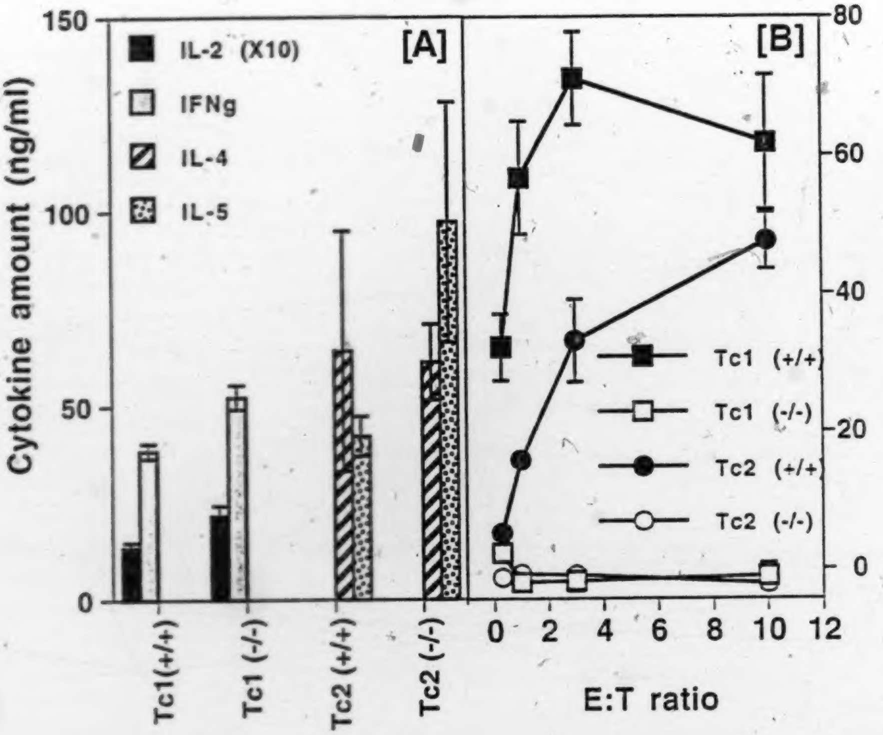


Figure 6.1. Perforin-deficient Tc1 and Tc2 cells produce similar cytokines as their normal counterparts, but do not mediate rapid killing of target cells. Naive spleen CD8 T cells ($H2^b$) derived from normal (+/+) or perforin-deficient (-/-) mice were stimulated with M12.4.1 cells ($H2^d$) in the Tc1- or Tc2-differentiating conditions for 7 d. An aliquot of these cells were stimulated with ConA for 24 hr, and cytokines were tested in the supernatants by ELISA (A). Different ratios of Tc to ^{51}Cr labeled M12.4.1 target cells were placed together and cultured for 4 hr, supernatantsthen collected, radioactivity counted to measure specific killing of target cells (B). Each bar or point represents the mean ± SD of triplicated wells.

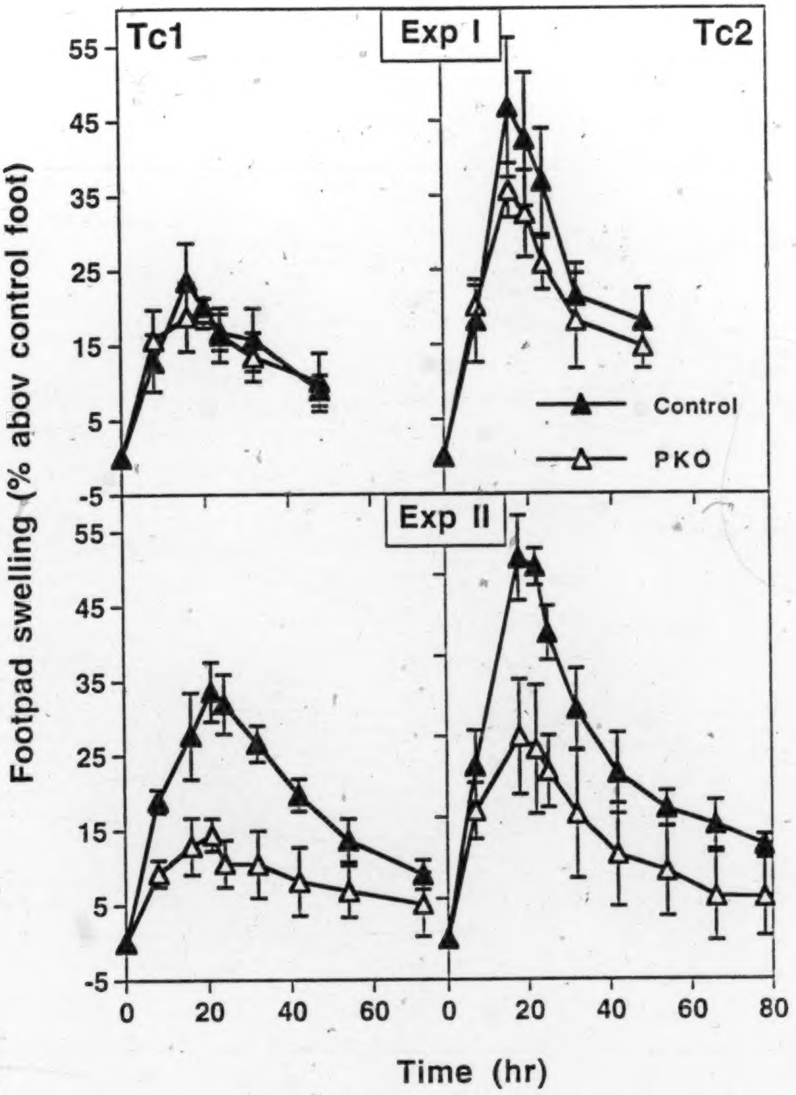


Figure 6.2. Perforin-deficient Tc1 and Tc2 cells induce similar or decreased footpad swelling compared to their normal counterparts. Equal numbers of Normal or perforin-deficient (PKO) Tc1 or Tc2 cells (10^6/mouse) were injected into the left hind footpads of naive BALB/c mice, the right hind footpads of these mice were injected with an equal volume of RPMI as controls. Each point represents the mean ± SD (n=4).

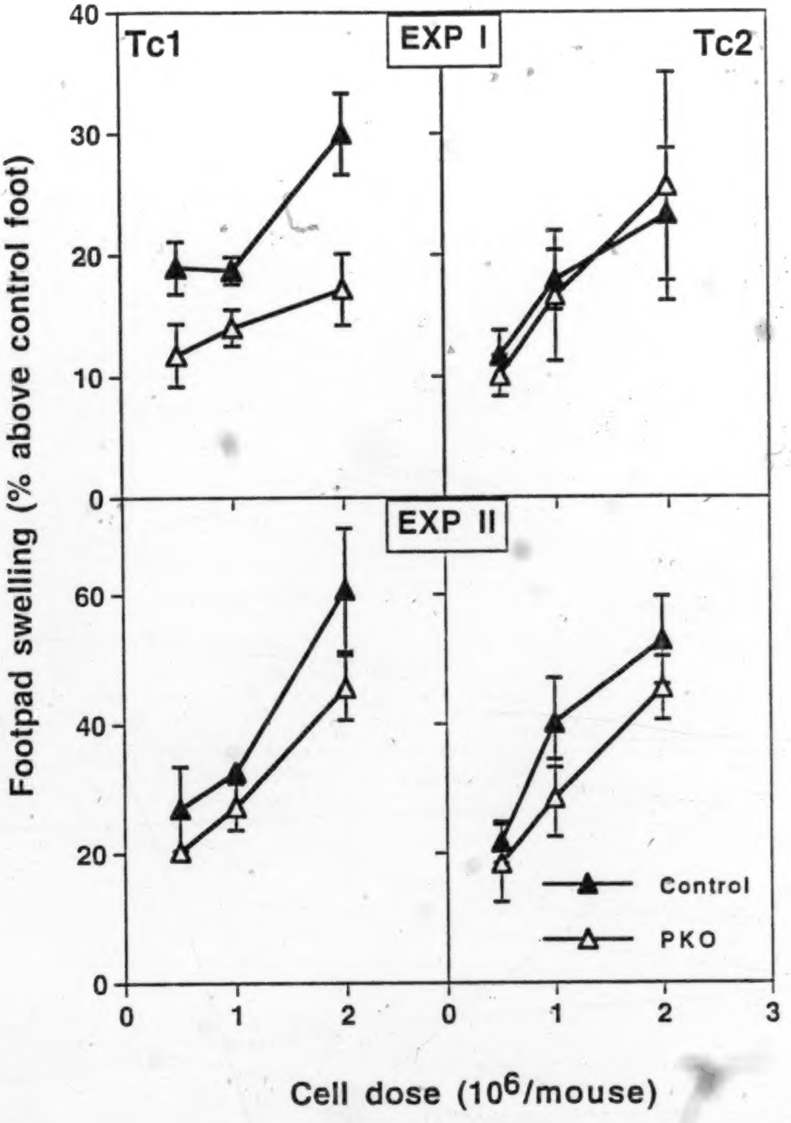


Figure 6.3. Perforin-deficient Tc1 and Tc2 cells induce cell dose-dependent footpad swelling. Different numbers of normal (Control) or perforin-deficient (PKO) Tc1 or Tc2 cells ($H2^b$-anti-$H2^d$) were injected into the left hind footpads of naive BALB/c mice ($H2^d$), the right hind footpads of these mice were injected with an equal volume of RPMI as controls. Footpad swelling was measured 22 hr after T cell injection. Each point represents the mean ± SD (n=4).

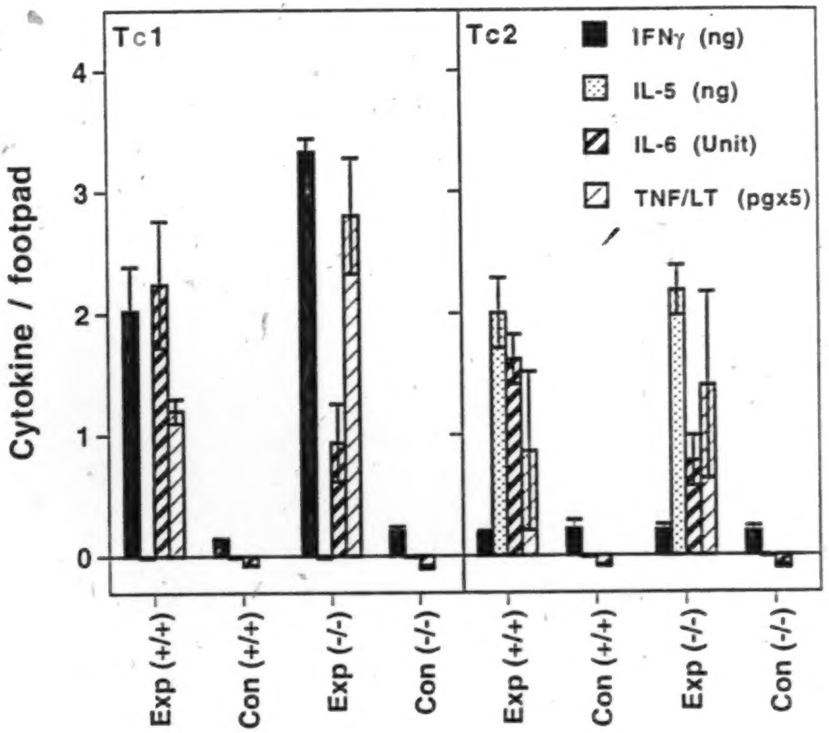


Figure 6.4. Similar cytokines are detected in DTH footpads injected with perforin-deficient Tc cells as in those injected with the normal counterparts. An equal number of perforin-deficient (-/-) or normal (+/+) Tc1 or Tc2 cells were injeted into the left hind footpads (Exp) of naive BALB/c mice to induce DTH, the right hind footpads of these mice were injected with an equal volume of RPMI as controls (Con). Footpads were collected 22 hr after T cell injection, and cytokines were detected in the footpad extracts by either ELISA (IL-5, IL-6 and IFNγ) or a bioassay (TNF/LT). Each bar represents the mean ± SD (Tc1, n=3; Tc2, n=4).

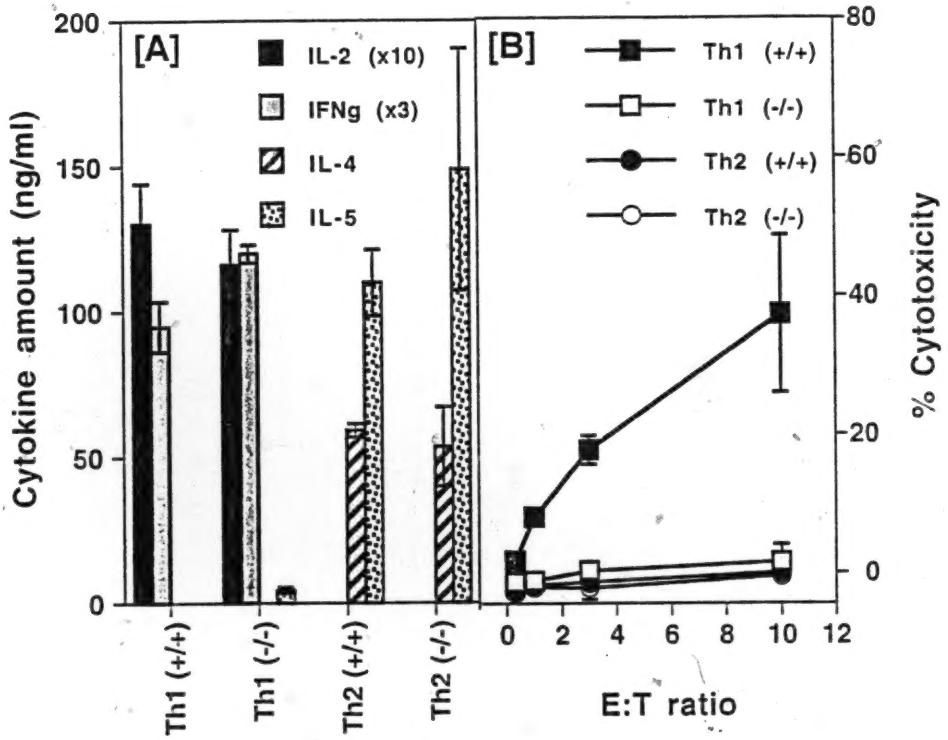


Figure 6.5. Perforin-deficient Th1 and Th2 cells produce similar cytokines as their normal counterparts, and Th1 but not Th2 cells express perforin-mediated cytotoxicity. Naive spleen CD4 T cells ($H2^b$) derived from either normal (+/+) or perforin-deficient (-/-) mice were stimulated with M12.4.1 cells ($H2^d$) in the Th1- or Th2-favoring conditions for 7 d. An aliquot of these cells were stimulated with ConA for 24 hr, and cytokines were tested in the supernatants by ELISA (A). Different ratios of Tc to ^{51}Cr labeled M12.4.1 target cells were placed together and cultured for 4 hr, supernatants were then collected, and radioactivity counted to measure specific killing of target cells (B). Each bar or point represents the mean ± SD of triplicated wells.

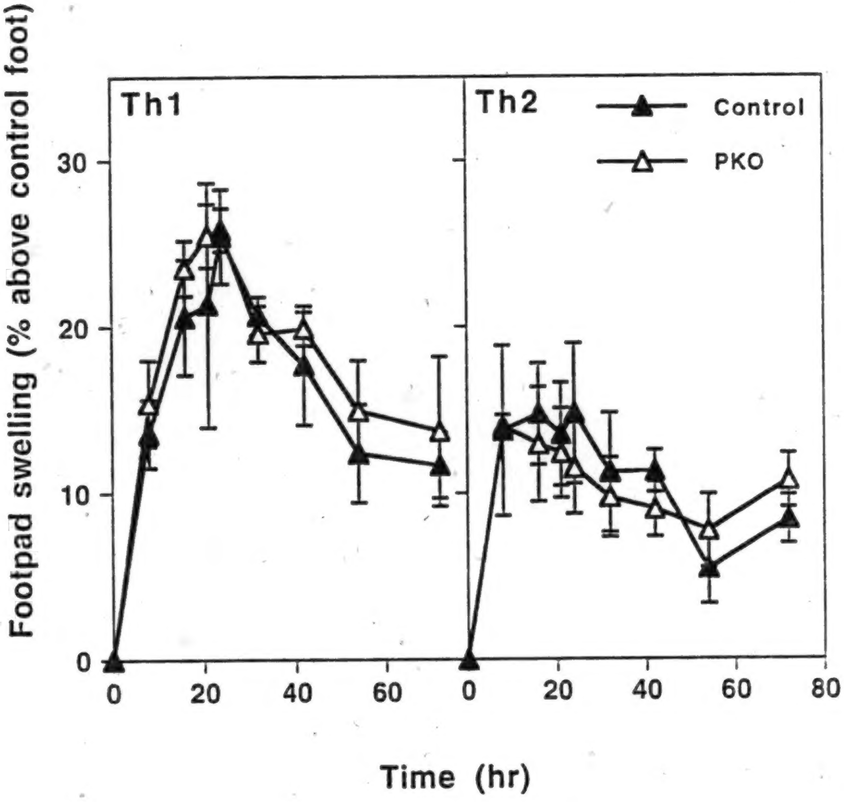


Figure 6.6. Perforin does not affect the abilities of either Th1 or Th2 cells to induce DTH. An equal number of normal (Control) or perforin-deficient (PKO) Th1 or Th2 cells ($H2^b$-anti-$H2^d$, 10^6/mouse) were injected into the left hind footpads of naive BALB/c ($H2^d$) mice, the right hind footpads of these mice were injected with an equal volume of RPMI as controls. Each point represents the mean ± SD (n=4).

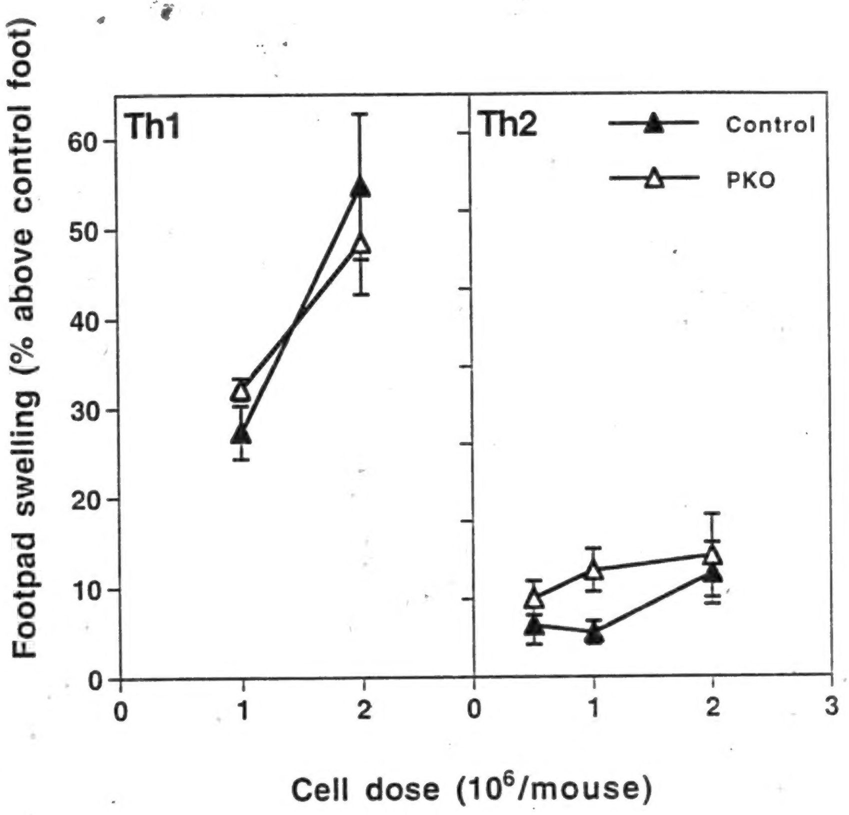


Figure 6.7. Both normal and perforin-deficient Th1, but not Th2, cells induce cell dose-dependent footpad swelling. Different numbers of normal (Control) or perforin-deficient (PKO) Th1 or Th2 cells ($H2^b$-anti-$H2^d$) were injected into the left hind footpads of naive BALB/c mice ($H2^d$). The right hind footpads of these mice were injected with an equal volume of RPMI as controls. Footpad swelling was measured 24 hr after T cell injection. Each point represents the mean ± SD (n=3).

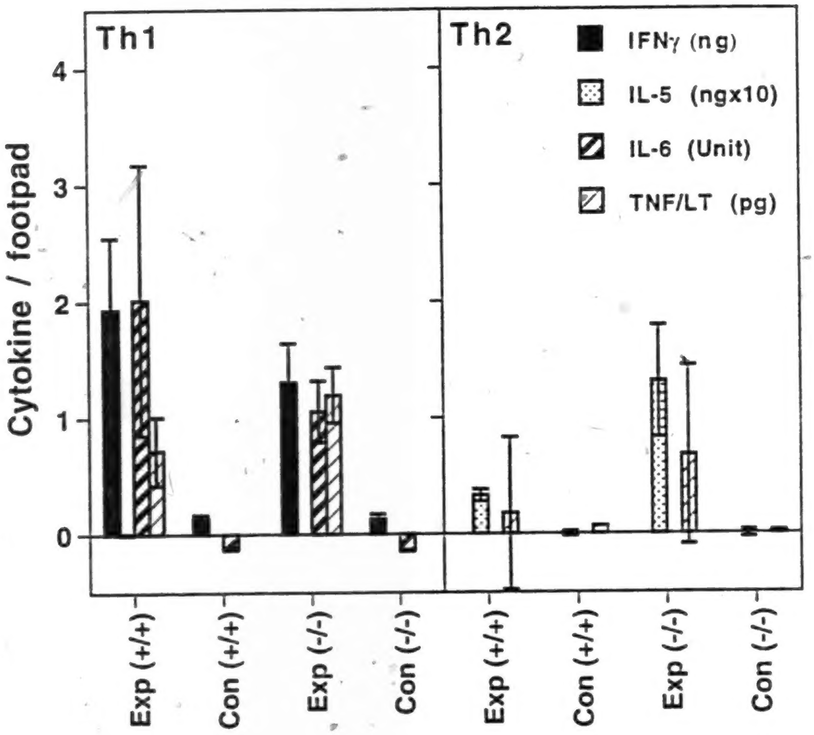


Figure 6.8. In vivo cytokine production in the footpads injected with normal or perforin-deficient Th1 or Th2 cells. An equal number of perforin-deficient (-/-) or normal (+/+) Th1 or Th2 cells were injected into the left hind footpads (Exp) of naive BALB/c mice, with their right hind footpads receiving an equal volume of RPMI as controls (Con). Twenty-four hr later, footpads were collected, and cytokines were detected from the tissue extracts by either ELISA (IL-5, IL-6 and IFNγ) or a bioassay (TNF/LT). Each bar represents the mean ± SD (n=3). (IL-6 was not measured for tissue extracts of Th2 injected footpads)

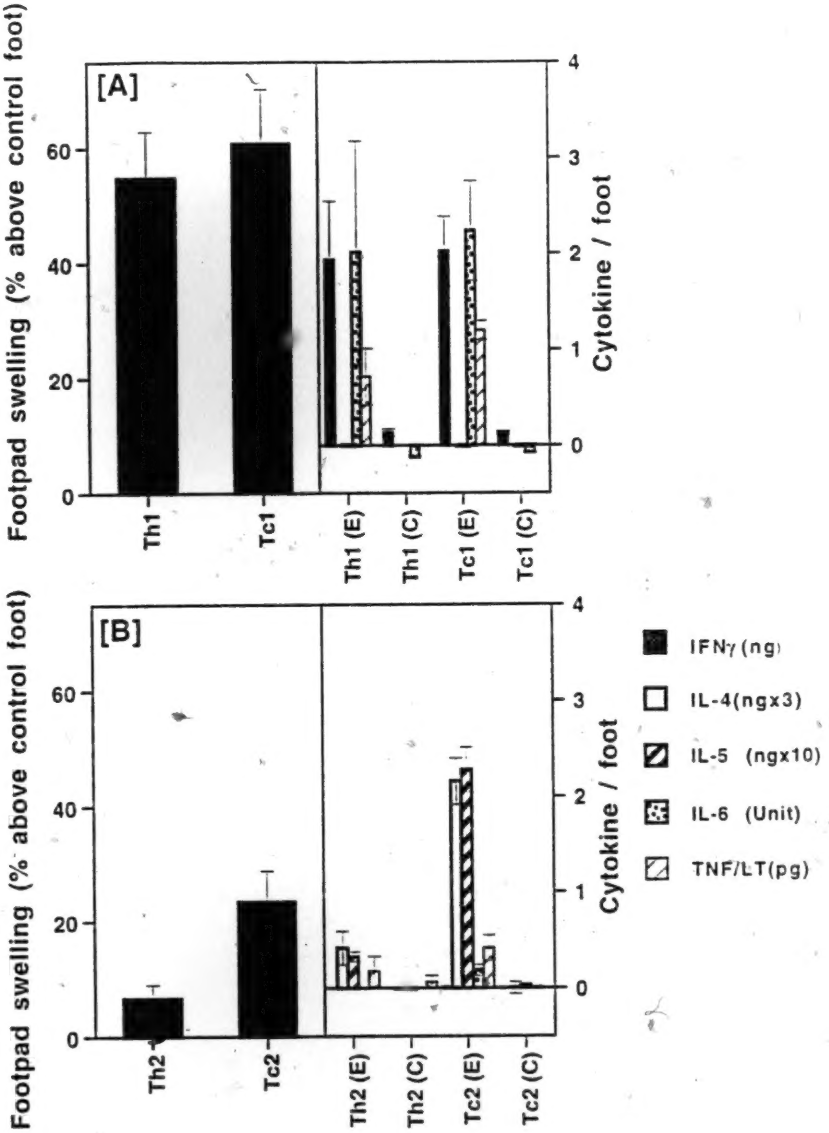


Figure 6.9. Th1 and Tc1 cells produce similar levels of cytokines in vivo, while Th2 cells produce lower levels of cytokine than Tc2 cells, which correlates with the less footpad swelling they induced. In two independent experiments (A and B), an equal number of Th1, Th2, Tc1 or Tc2 cells were injected into the left hind footpads of naive BALB/c mice. Twenty-two (Tc) or 24 (Th) hr later, the footpad swelling was measured and the footpads were collected. Cytokines were detected in the footpad extracts by ELISA (IFNγ, IL-4, IL-5 and IL-6) or a bioassay (TNF/LT). Each bar represents the mean ± SD (n=3).

Chapter VII.

General discussion and future projects

A. General discussion:

Since the discovery of the Th1 and Th2 cytokine patterns among CD4 T cells, large numbers of studies have concentrated on the physiological significance of these cytokine subsets in immunity. Based on results of many animal studies and human diseases, Th1 and Th2 cells play different roles in immune regulation. Th1 cells and their cytokines, IL-2 and IFNγ, are often associated with cell-mediated immune responses, such as DTH, while Th2 cells and their cytokines are more active in helping antibody production and enhancing allergic responses (Mosmann and Sad, 1996; Gemmell and Seymour, 1994). Furthermore, Th1 and Th2 cells are cross-regulated through their cytokines, which might partially explain the reciprocal suppression of DTH and Ab responses often seen in vivo. To test the counter regulation between Th1 and Th2 cells and their cytokines in vivo, the suppressive function of one of the Th2 cytokines, IL-10, on DTH was studied. However the correlation between T helper subsets and different classes of immune response is not absolute. The Th1 cytokine, IFNγ, can provide B cell help and induce an Ab isotype switch to IgG2a. This isotype of Ab is effective for fixing complement, so IFNγ can be an important mediator in Ab responses. On the other hand, Th2 cells also mediate inflammation or DTH responses. Therefore the immune regulation mediated by Th1 and Th2 cells is more complicated than a clear dichotomy.

DTH is an in vivo manifestation of cellular immunity, which is often considered to be a Th1 rather than Th2 response. Th1 cytokines e.g. IL-2 and IFNγ, can be detected from local DTH sites (Tsicopoulos et al., 1992) or from the draining lymph nodes (Heinzel et al., 1989). Antigen-specific Th1 cells can be readily isolated from sites of

DTH responses in human diseases (Del Prete et al., 1994). PPD induces a DTH response in primed individuals, and human T cells specific for PPD mostly express the Th1 cytokine pattern (Del Prete et al., 1991b; Haanen et al., 1991). Resting Th1 clones induce footpad DTH in naive mice when locally transferred with corresponding antigens, and the DTH reaction is partially regulated by IFNγ (Cher and Mosmann, 1987; Fong and Mosmann, 1989). *Leishmania major*-specific Th1 clones adoptively transfer both DTH and protection to *L. major* infection (Scott et al., 1988), which can only be cured through cell-mediated immune responses. All these results demonstrated that Th1 cells and their cytokines are important activators of DTH responses.

Th2 cells are not normally associated with DTH reactions in many diseases (Mosmann and Sad, 1996; Gemmell and Seymour, 1994), in fact Th2 cytokines, especially IL-10, showed inhibitory effects on DTH and other inflammations. The anti-inflammatory effect of IL-10 has been shown by many studies, as well as the results in this thesis (see chapter IV). IL-10 inhibits the production of IFNγ and TNFα (Fiorentino et al., 1989; Fiorentino et al., 1991a), and accordingly may suppress the cytokine-dependent activation of endothelial cells and the initiation of local inflammation. IL-10 also suppresses the functions of macrophages (Fiorentino et al., 1991a; Gazzinelli et al., 1992b; Oswald et al., 1992a; Cunha et al., 1992) and neutrophils (Wang et al., 1994; Kasama et al., 1994). Therefore, it could reduce the tissue swelling and tissue damage caused by the activation of cells in DTH. IL-10 was produced during DTH (Chapter IV) or other inflammatory reactions (Kucharzik et al., 1995), and was responsible for quenching of inflammation (Ferguson et al., 1994). This was further confirmed by the extremely severe DTH reaction developed in IL-10 deficient mice (Berg et al., 1995). Furthermore, IL-10 and IL-4 synergized to inhibit DTH (Powrie et al., 1993), suggesting that Th2 cells, by producing a mixture of Th2 cytokines, could be more potent than IL-10 alone in DTH suppression.

Although DTH can not be normally induced in Th2 dominated immune responses, and Th2 cytokines inhibit DTH, Th2 cells have been reported to be involved in some DTH reactions. Both Th1 and Th2 cytokines were detected at either mRNA or protein levels in the DTH sites (Buchanan and Murphy, 1993; Ng et al., 1995). Ag-specific Th1 and Th2, or Th0 cells were isolated from biopsies of DTH tissues (MacPhee et al., 1993; Picker et al., 1995). These results suggested that both Th1 and Th2 cells could participate in local DTH reaction. But whether these Th2 cells functioned as positive or negative regulators in these DTH reactions was unclear.

The direct contribution of Th2 cells and their cytokines to DTH responses has been studied. Th2 cells induced IL-4-dependent secondary granulomas towards *Schistosoma* egg Ags (SEA), and this reaction involved heavy infiltration of eosinophils (Wynn et al., 1993). Pulmonary granulomas induced by beads coated with SEA were caused by Th2 cells as well (Chensue et al., 1994). These granulomas were augmented by anti-IFNγ Ab treatment, suggesting that endogenous IFNγ was a negative regulator of the granulomas (Chensue et al., 1995b). Preactivated Th2 bulk cultured cells induced an IL-4 dependent DTH-like footpad inflammatory response (Muller et al., 1993), which was infiltrated predominantly by both neutrophils and macrophages (Muller et al., 1995a). Furthermore, IL-4 was required for local migration of DTH effector T cells (Dieli et al., 1994) in contact hypersensitivity. Anti-IL-4 treatment not only suppressed the DTH induced by trinitrochlorobenzene (TNCB), but also blocked the ability of T cells to transfer DTH to naive mice (Salerno et al., 1995). All these results suggested positive roles of Th2 cells and their cytokines in certain DTH responses. Thus, the fact that Th2 cells inhibited DTH through the actions of their anti-inflammatory cytokines does not necessarily mean that these cells themselves are not able to induce inflammation through other mechanisms.

CD8 T cells are the Ag-specific cytolytic T cells whose major function is to kill virus-infected cells, tumors and allografts. The mechanisms of CD8 T cell-mediated cytotoxicity involve both perforin-dependent and Fas-mediated pathways (Liu et al., 1995). CD8 T cells mediate DTH responses towards MHC class I restricted Ags such as viral Ags (Liew, 1982; Kundig et al., 1992); they are also the major mediators of certain contact hypersensitivity reactions (Bour et al., 1995; Xu et al., 1996). Furthermore, CD8 T cells can adoptively transfer footpad DTH (Lin and Askonas, 1981). All these results demonstrated an important role of CD8 T cells in certain DTH responses. Like CD4 T cells, CD8 T cells produce multiple cytokines when activated. Recently distinct cytokine patterns similar to those of the Th1 and Th2 cells were generated in vitro among CD8 T cells (Croft et al., 1994; Sad et al., 1995), or isolated in vivo from normal or infected individuals (Maggi et al., 1994; Birkhofer et al., 1996). But unlike Th1 and Th2 cells, the in vitro generated Tc1 and Tc2 cells showed similar functions in both CTL and B cell help assays. Most of the in vivo isolated Tc2 cells expressed significantly less cytotoxicity than Tc1 cells (Birkhofer et al., 1996; Maggi et al., 1994), but the mechanism which caused the difference is not clear. The potential physiological functions of Tc1 and Tc2 cells need to be further investigated. Therefore, the abilities of Tc1 and Tc2 cells to induce DTH, and the contribution of their perforin-mediated cytotoxicity to DTH reaction have been tested.

Studies in this thesis (chapter V and VI) investigated the abilities of Tc1 and Tc2 cells to induce DTH. Despite their distinct cytokine patterns both in vitro and in vivo, Tc1 and Tc2 cells induce similar DTH-like inflammation when transferred locally. Tc1 and Tc2 cells were also able to preferentially migrate into inflammatory sites. Therefore, both cytokine subsets of CD8 T cells have the potential to mediate DTH during immune responses. Furthermore, this study confirmed that DTH-like inflammatory responses could develop in both Tc1 (Th1) and Tc2 (Th2) cytokine environments. Although Th1 and Th2 mediated DTH were regulated by IFNγ and IL-4 respectively, the major regulatory factors of Tc1 and Tc2 cells were not as obvious. The magnitude of Tc1 induced DTH was not correlated with the in vivo levels of IFNγ nor was it inhibited by IFNγ Ab, suggesting that IFNγ may not be a critical factor for Tc1 to induce DTH. DTH induced by Tc2 cells was not significantly inhibited by anti-IL-4 Ab, suggesting it was not IL-4-dependent. Perforin-mediated cytotoxicity sometimes affected the magnitude of DTH induced by both Tc1 and Tc2 cells, although it was not crucial for the DTH. Collectively these results suggested that adoptively transferred DTH induced by CD8 T cells may be regulated by multiple factors.

IL-10 and IL-4 are believed to be anti-inflammatory cytokines, then why do Th2 and Tc2 cells induce locally transferred footpad DTH? One explanation could be the kinetics of different cytokine production. IL-10 was often produced at a relatively late stage of activation, indicating its regulatory role in the later stage of the immune responses (Yssel et al., 1992). When Th2 (Tc2) cells were activated, other cytokines such as IL-4, IL-5 and TNFα may be produced earlier. IL-4 expresses dual effects on inflammatory responses depending on the cytokine environment. In the presence of IL-1 and TNFα, IL-4 activates endothelial cells (Masinovsky et al., 1990; Briscoe et al., 1992) and induces strong tissue damage (Hernandez-Pando and Rook, 1994). Furthermore, IL-4 is a chemotactic factor for eosinophils during inflammation (Tepper et al., 1992). Therefore, IL-4 could initiate and enhance a DTH reaction. Secondly, although IL-4 and IL-10 show synergy in DTH inhibition, there may not always be enough IL-10 expressed in vivo. IL-10 was detected in DTH induced by a Th1 clone, but not in Tc1 and Tc2 DTH footpads at either early (8 hr) or late (24 to 40 hr) stages. This finding suggested that either the CD8 T cells did not produce IL-10 in the footpads or the production was very low. Since for DTH inhibition, a relatively large amount of IL-10 was required, the low IL-10 production may not be sufficient to inhibit DTH induced by Tc1 or Tc2 cells. Thirdly, instead of inhibiting DTH, IL-10 can also be a positive mediator for inflammation. IL-10 is a chemotactic factor for CD8 T cells (Jinquan et al., 1993), and transgenic expression of IL-10 in the islets of Langerhans led to a pronounced cellular infiltration in the pancreas (Wogensen et al., 1993). However, what determines the different functions of IL-10 expressed during inflammation remains to be resolved. But these observations suggested that cytokines may function differently in vivo depending on the microenvironments, and Th2 cytokines may not always be anti-inflammatory mediators,. Therefore local administration of these cytokines may not always be a good strategy for inhibition of inflammatory responses.

Although Th2 and Tc2 cells produced similar patterns of cytokines, Tc2 cells were much more active in mediating adoptively-transferred DTH responses. This result suggested that additional factors other than their anti-inflammatory cytokines were preventing Th2 cells from inducing DTH. Resting Th2 cells were poor in DTH induction (Cher and Mosmann, 1987), but when preactivated these cells induced comparable levels of footpad swelling to those induced by Th1 cells (Muller et al., 1995b and Figure 5.5). The requirement for preactivation in vitro suggests that Th2 cells were not fully activated in footpads. This suggestion was further confirmed by the low cytokine levels detected in footpads injected with resting Th2 cells compared to those in Tc2 footpads (Figure 6.9). Th2 and Tc2 cells are activated by MHC class II and class I positive APCs respectively. Since there are more MHC class I than class II positive cells in the footpads, Tc2 cells could receive more stimulation than Th2 cells. Although Th1 and Th2 cells both are activated by MHC class II positive cells, Th1 cells were more activated than Th2 cells in the footpads. These results suggested that Th2 cells may require different types of APCs in vivo, or need more costimulations. But it needs to be further investigated whether the lack of activation of Th2 cells in footpad tissues is also true for in vivo generated Th2 cells during infectious diseases.

Besides the in vivo activation, the ability of T cell homing to local challenged tissue also determines if a particular T cell subtype is able to induce DTH in immune responses. Local inflammation stimulates the expression of adhesion molecules on vascular endothelial cells, and these adhesion molecules together with chemokines bring about the extravasation of circulating T cells (Pober and Cotran, 1990; Shimizu et al., 1992). The migration of both Tc1 and Tc2 cells into the tissue where inflammation was initiated suggested their ability to mediate DTH in immune responses. Nevertheless, among three independent experiments Tc1 showed consistently stronger local migration than Tc2 cells. These results indicated that Tc1 may be more effective than Tc2 cells in inducing a DTH. Accordingly a recent study showed that cultured Th1 but not Th2 bulk populations can efficiently enter inflamed tissue sites, such as sensitized skin or arthritic joints (Austrup et al., 1997). The preferential migration of Th1 cells was mediated by P- and E-selectin, and only Th1 cells can bind to these adhesion molecules. This study indicated that adhesion mechanisms provided an additional level of regulation for Th1 and Th2 cells in certain immune responses. However further studies are needed to test if the in vivo isolated Tc1 and Tc2 or Th2 and Th2 cells also show migration difference.

Although Tc2 and activated Th2 cells have the potential to mediate DTH, in many infectious diseases DTH was not induced in a Th2 type of response (Mosmann and Sad, 1996). Thus, factors other than Th2 cells and their cytokines might negatively regulate DTH in a Th2 dominated response. One of the factors that controls the unresponsiveness of DTH could be DTH suppressor T cells (Howard et al., 1981). Depending on the system studied, DTH suppressor T cells can be either CD4 or CD8 cells, and can function at either the induction or the effector phase of DTH responses. T suppressor cells function through their secreted Ag-specific suppressor factors (Dorf and Benacerraf, 1984). However, despite the long history of study, the mechanisms of the suppressor factors are still poorly understood. It has been demonstrated that many suppressor factors contained two linked chains, one directly recognized the Ags while the other was I-J$^+$ that may control the genetic restriction (Dorf and Benacerraf, 1984). Instead of binding to Ags, a few suppressor factors were found carrying anti-idiotype specificity. Therefore, they might function via an idiotype-anti-idiotype interaction (Nash and Gell, 1981). A modified soluble form of TCR α chain was also reported as a Ag-specific T suppressor factor (Zheng et al, 1989), although whether this type of factor is involved in DTH suppression is not known. Understanding of the relationship between Th2 dominated responses and DTH suppressor cells, and the further characterization of these cells and their soluble factors would provide important information to help resolve the mechanisms of DTH unresponsiveness in Th2 responses.

Both Tc1 and Tc2 cells (also some T helper cells) express perforin-mediated cytotoxic activity. For CD8 T cells this is the major pathway of their cytotoxic activity. However, perforin-deficient Tc1 and Tc2 cells were able to transfer DTH, although sometimes the magnitude of the responses was lower. These findings suggest that cytotoxicity may contribute to DTH reactions induced by Tc cells, but it is not a critical factor. Other studies have shown that allograft rejection and CD8 T cell-mediated insulitis were induced in perforin-deficient mice to a similar extent as in normal mice (Schulz et al., 1995; Kagi et al., 1996). Together these results suggested that perforin-dependent cytotoxicity was not an essential factor for CD8 T cells to mediate inflammation. However, the influence of perforin on DTH induced by Tc1 and Tc2 cells did not seem to be equal. The lack of perforin affected the footpad swelling induced by Tc1 cells more dramatically than that induced by Tc2 cells. This result indicated that the mechanism of DTH induction by Tc1 and Tc2 cells may be different. Perforin-mediated cytotoxicity maybe involved more in the inflammatory response induced by Tc1 than by Tc2 cells.

Th1 DTH often serves as the defense mechanism against infections (Mosmann and Sad, 1996). The physiological roles of the Th2 or Tc2 cell-mediated DTH are poorly understood. In mouse *Schistosoma* infection, macrophages are thought to be important effector cells of protective immunity (Sher et al., 1992). Vaccine-induced resistance in vivo was substantially reduced in inbred strains with genetic defects in macrophage effector function, and macrophages activated by IFNγ treatment are potent killers of *schistosome* larvae in vitro. However, a Th2 cell-dominated response was normally developed during the time of *Schistosoma* egg deposition (Xu et al., 1991). Since macrophage activities were suppressed by Th2 cytokines such as IL-4 and IL-10 (Hamilton and Adams, 1987; Fiorentino et al., 1991a), the Th2 response could also suppress the protection against *Schistosoma* infection. Th2 cells predominantly regulated the secondary granulomas induced by *schistosome* eggs (Scott et al., 1989), but these granulomas are mainly the cause of pathology rather than a defense mechanism.

The ability of both Tc1 and Tc2 cells to mediate inflammation could be an advantage in responses to different pathogens. CD8 T cells are major effectors in anti-viral immunity. Th2 type of responses were often developed in certain virus infections, e.g. in mouse acquired immunodeficiency syndrome (MAIDS) (Gazzinelli et al., 1992a) and respiratory syncytial virus (RSV) infection (Openshaw, 1995). Furthermore, a Th1 to Th2 response switch was observed during human immunodeficiency virus (HIV) infection (Clerici and Shearer, 1993). Tc2 cells were generated in a Th2 biased response (Maggi et al., 1994; Coyle et al., 1995). Therefore, Tc2 cells could be developed during a Th2 dominated anti-viral immunity. Thus, the ability of both Tc1 and Tc2 cells to mediate DTH may benefit the host defense against viruses causing both Th1 and Th2 dominated immune responses. The Tc cell-mediated DTH may enhance the clearance of the infected target cells and the cell bound virus by recruiting Ag-nonspecific phagocytic cells. This event could synergize with the CTL killing in clearing the infections.

In the adoptively transferred footpad DTH, although similar edema and neutrophil/macrophage infiltration were induced by Tc1 vs Tc2 (our study), and Th1 vs Th2 (Muller et al., 1995b) cells, it does not mean that the final effects initiated by these two types of Th (Tc) cells are the same. The physiological functions of DTH are expressed through the activation of Ag-nonspecific effector cells especially macrophages and neutrophils. During the effector stage of a DTH, Th1 (Tc1) and Th2 (Tc2) cytokines express different effects on macrophages and neutrophils. IL-4 and IL-10 inhibit, whereas IFNγ activates the functions of these cells. Furthermore, IL-5 preferentially activates eosinophils. Therefore different activation states of these infiltrated cells may occur in DTH mediated by Th1 vs Th2, Tc1 vs Tc2 cells. This was proved recently that different activation status of macrophages were demonstrated in Th1 and Th2 cytokine-mediated granulomas (Chensue et al, 1995a). Since the potential functional differences of the Ag-nonspecific effector cells will affect the clearance of pathogens, DTH induced by Th1(Tc1) and Th2(Tc2) cells may result in different disease progression and different fates of the hosts in certain cases.

Besides the potential difference in pathogen clearance, DTH induced by Th1 (Tc1) and Th2 (Tc2) cells may also cause different tissue damage. The anti-inflammatory cytokines produced by Th2 (Tc2) cells may cause less tissue damage in DTH induced by these cells, but it has been reported that IL-4 and TNFα cause stronger tissue damage than that mediated by Th1 cytokines (Hernandez-Pando and Rook, 1994). Since Th2 (Tc2) cells may not efficiently activate macrophages in a local DTH site, accordingly this DTH-like inflammatory response may not be an efficient defense mechanism in controlling intracellular pathogens. Therefore the DTH responses mediated by Th2 (Tc2) cells may mainly cause tissue damage, and accordingly be responsible for the pathogenesis of certain diseases. However, the physiological roles of Th2 (Tc2) DTH need to be further investigated in infectious disease models.

It has been a century since Koch first described a DTH reaction induced by tuberculin. Through a long history of extensive research, many of the aspects and mechanisms of this reaction have been understood and used to benefit our fight against many diseases. Studies presented in this thesis provide further information about the potential roles of cytokine T cell subsets in DTH reaction and the regulatory effects of their soluble products on DTH. I show here that IL-10 suppressed DTH, probably through its inhibition of Th1 and macrophage functions in vivo; DTH could be induced by local transfer of both Tc1 and Tc2 cells, and the DTH was not solely dependent on the characteristic cytokines of Tc1 or Tc2 cells; perforin-dependent cytotoxicity enhance the extent of DTH induced by Tc1 and Tc2, especially Tc1 cells, but it was not absolutely required for DTH reactions; perforin has no detectable effect on Th1 and Th2 cells to induce DTH. DTH is a cascade of in vivo processes that involves multiple cell types and a large variety of regulatory soluble molecules. Despite the long history of study, there are still lots of unknowns about the DTH process that need to be further investigated. Therefore there is still a long way to go before we finally understand DTH.

B. Potential future projects:

DTH has been often considered as a Th1 dominated response. However, recent studies (including the work in this thesis) have demonstrated that Th2 and Tc2 cells are able to induce similar cellular infiltration and vascular permeability as Th1 and Tc1 cells respectively, suggesting that Th2 and Tc2 can also be DTH effector cells in vivo during immune responses. But why is DTH not normally mounted in a Th2 dominated response? Our preliminary results suggested a couple of possible explanations. The failure to mount a DTH or DTH-like inflammatory response in most Th2 mediated immunities could be due to either the poor activation of Th2 cells in certain tissues; or their poor ability to migrate into inflamed tissues; or to the suppression of DTH by either Th2 cells and their cytokines or cells other than Th2 type by undetermined mechanisms. In the adoptively transferred DTH model, the Th2 and Tc2 cells used so far were all allo-responsive. To understand their activity to induce DTH during infection, pathogen Ag-specific Th and Tc cells need to be generated and their functions studied in mouse infectious models.

The well defined mouse *L. major* infection system provides a good model to study the in vivo functions of different T cytokine subsets. It has been well documented that after infection, the resistant strain (e.g. C57BL/6J) develops a Ag-specific Th1 and DTH responses, while the susceptible strain (e.g. BALB/c) generates Ag-specific Th2 cells and Ab response, but no DTH (Locksley and Louis, 1992). Using this model, we can study the DTH induction by Ag-specific T subsets and their functions in disease protection. *L. major* specific Th2 cell lines or clones can be isolated from the draining lymph nodes (LN) of infected BALB/c mice (Holaday et al., 1991). Th1 cell lines or clones may also be isolated from these mice (Holaday et al., 1991), but more likely they will be established from BALB/c mice being treated with anti-IL-4 (Sadick et al., 1990), or sublethal irradiation (Howard et al., 1981), or IL-12 (Seder et al., 1993) before *L. major* infection. *L. major* Ag-specific Tc1 cells may be generated from the draining LN of infected BALB/c mice with pre-anti-CD4 treatment (Hill et al., 1989), and Tc2 from infected BALB/c mice with both anti-CD4 and IL-4 treatments. All these cell lines or clones could be cultured in vitro, and their Ag specificity checked. The Th1 and Th2 that recognize the same pathogen proteins could be matched as pairs or groups, and the same done for Tc1 and Tc2 cells. The Ag-specificity match may not be easy, since different *L. major* Ags preferentially induced either Th1 or Th2 cells (Scott et al., 1989). The in vivo functions could be compared between different cell types within each group. To test their ability to mediate the effector stage of *L. major* Ag-specific DTH, these cells will be transferred into syngeneic SCID mice either locally into the footpads with corresponding Ags, or systemically with Ag challenging in one footpad. In addition, these cells could be radiolabeled and their migration ability to an inflammatory site could be measured by radioactivity counting. Furthermore the physiological functions of these cells against *L. major* infection will be investigated in syngeneic SCID mice. After T cell transfer, these mice will be infected with *L. major* and their disease progress will be monitored by either the size of lesion or by parasite count. The ability of each cell type to induce DTH and to provide protection will be compared. I believe that this set of experiments will provide information about the potential ability of Ag-specific Th2 and Tc2 subsets to mediate a DTH response during an infectious disease, and the function of these cells in providing *L.major* protection.

It has been reported for decades that CD4 T cells in BALB/c mice suppressed *L. tropica* (maybe also *L. major*) induced DTH (Howard et al., 1981). The existence of these cells maybe one of the reasons for the unrestrained disease progression. This hypothesis has also been supported by the finding that depletion of CD4 T cells results in DTH response and resistance to *L. major* infection in BALB/c mice (Hill et al., 1989). However the cytokine pattern of the suppressor cells remains unclear. They could be Th2 cells inhibiting DTH through IL-10 or other mediators, or it is also possible that they are other cell types. Further characterization of these suppressor cells and their secreted factors will help us to understand the mechanism of DTH suppression. Alternatively using L. major specific T cytokine subsets, we can also test the possible inhibitory effect of Th2 or Tc2 cells on Th1 or Tc1 mediated DTH. The Ag specificity matched Th1 and Th2 cells or Tc1 and Tc2 cells or other combinations will be co-transferred into syngeneic SCID mice, and the mounting of the effector stage of *L. major* Ag-specific DTH will be monitored to test the potential inhibitory effects of Th2 and Tc2 cells. In addition, Th2 or Tc2 cells will also be transferred into the pre-anti-IL-4 treated, *L. major* resistant BALB/c mice to test their inhibitory effects on DTH or on disease protection. These experiments will provide information about the direct effects of Th2 or Tc2 cells on Th1 or Tc1 mediated DTH and *L. major* protection.

The recent generation of the Tc2 phenotype and the discovery of its in vivo existence in both normal and infected individuals make it important to understand the biological significance of this cell type in immune responses. Allo-reactive Tc1 and Tc2 cells are able to locally transfer DTH, and can preferentially migrate into an inflammatory site, suggesting they may induce DTH during immune responses. But whether this is also the case for pathogen-specific Tc cells during infection still needs to be further investigated. Beside the *L. major* system discussed earlier, the mouse influenza infection is another good model for the study. The peptides of influenza that can stimulate protective CD8 T cells have been identified (Townsend et al., 1986). Using these peptides, influenza antigen-specific Tc1 and Tc2 cells can be generated in vitro from naive or infected mouse splenocytes. These Tc1 or Tc2 cells will then be systemically transferred into irradiated syngeneic naive mice, and the protection they provide against influenza virus infection can then be tasted. Furthermore, by challenging these T cell-reconstituted mice with the influenza peptides in their footpads, the ability of the peptide-specific Tc1 and Tc2 to induce DTH will also be tested and the correlation between DTH induction and protection will be studied.

Besides the role of disease protection, it is also important to understand the possible pathogenesis caused by Tc1 and Tc2 cells. Using the LCMV peptide-specific T cell receptor (TCR) transgenic (Pircher et al., 1989) and the insulin promoter directed LCMV glycoprotein (GP) transgenic (Ohashi et al., 1993) systems, we can study the activity of peptide-specific Tc1 and Tc2 cells in causing inflammation. LCMV peptide-specific Tc1 and Tc2 cells will be generated form the TCR transgenic mice in vitro. It has been reported that when activated and adoptively transferred, CD8 T cells from the LCMV-specific TCR transgenic mice can cause both insulitis and diabetes in LCMV GP transgenic mice (Kagi et al., 1996). By transferring the in vitro-generated Tc1 and Tc2 cells derived from the LCMV peptide-specific TCR transgenic mice into LCMV GP transgenic mice, the ability of these cells to induce insulitis and diabetes can be studied. We will look at the cellular infiltration of the local inflammatory area quantitatively by cell extraction and staining, and we will also check the severeness of the β islet cell destruction by measuring blood glucose levels of these mice.

Overall, I believe that the studies described above will provide important information about the regulatory mechanisms of DTH in a Th2 type responses, and the physiological roles Tc1 and Tc2 cells might play in protecting against virus or intracellular protozoan infection and in causing DTH or other inflammations. The more we understand DTH responses and their regulation, the more likely we can manipulate the immune system to either enhance the protective, or to suppress the harmful DTH responses.

Chapter VIII. Bibliography

Abramson, S.L. and Gallin, J.I. (1990). IL-4 inhibits superoxide production by human mononuclear phagocytes. J. Immunol. *144*, 625-630.

Actor, J.K., Shirai, M., Kullberg, M.C., Buller, R.M.L., Sher, A., and Berzofsky, J.A. (1993). Helminth infection results in decreased virus-specific CD8$^+$ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. Proc. Natl. Acad. Sci. USA *90*, 948-952.

Adams, D.O. and Hanna, M.G. Jr. (1984) Macrophage activation, Contemp. Top. Immunobiol., *13*, 1-262

Ahmed, A.R. and Blose, D.A. (1983). Delayed-type hypersensitivity skin testing. A review. Archives of Dermatology *119*, 934-945.

Alderson, M.R., Pike, B.L., and Nossal, G.JV. (1987). Single cell studies on the role of B-cell stimulatory factor 1 in B-cell activation. Proc. Natl. Acad. Sci. USA *84*, 1389-1393

Anderson, A.O., and S. Shaw (1994) Lymphocyte trafficking. In Clinical Immunology. R.R. Rich, T.A. Fleisher, B.D. Schwartz, W.T. Shearer, and W. Strober, editors. Mosby Year Book, St. Louis

Araneo, B.A., Dowell, T., Moon, H.B., and Daynes, R.A. (1989). Regulation of murine lymphokine production in vivo. Ultraviolet radiation exposure depresses IL-2 and enhances IL-4 production by T cells through an IL-1-dependent mechanism. J. Immunol. *143*, 1737-1744.

Arock, M., Zuany-Amorim, C., Singer, M., Benhamou, M., and Pretolani, M. (1996). Interleukin-10 inhibits cytokine generation from mast cells. Eur. J. Immunol. *26*, 166-170.

Askenase, P.W. (1976). Cutaneous basophil hypersensitivity uncovered in the cell transfer of classical tuberculin hypersensitivity. J. Immunol. *117*, 741-747.

Askenase, P.W. (1992). Delayed-type hypersensitivity recruitment of T cell subsets via antigen-specific non-IgE factors or IgE antibodies: relevance to asthma, autoimmunity and immune responses to tumors and parasites. Chemical Immunology *54*, 166-211.

Askenase, P.W., Van Loveren, H., Kraeuter-Kops, S., Ron, Y., Meade, R., Theoharides, T.C., Nordlund, J.J., Scovern, H., Gerhson, M.D., and Ptak, W. (1983). Defective elicitation of delayed-type hypersensitivity in W/Wv and SI/SId mast cell-deficient mice. J. Immunol. *131*, 2687-2694.

Austrup, F., Vestweber, D., Borges, E., Lohning, M., Brauer, R., Herz, U., Renz, H., Hallmann, R., Scheffold. A., Radbruch, A. and Hamann A. (1997). P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. Nature. *385*, 81-83.

Banchereau, J., de Paoli, P., Valle, A., Garcia, E., and Rousset, F. (1991). Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science *251*, 70-72.

Belosevic, M., Finbloom, D.S., Van Der Meide, P.H., Slayter, M.V., and Nacy, C.A. (1989). Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with Leishmania major. J. Immunol. *143*, 266-274.

Berg, D.J., Leach, M.W., Kuhn, R., Rajewsky, K., Muller, W., Davidson, N.J., and Rennick, D. (1995). Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. J. Exp. Med. *182*, 99-108.

Bergstresser, P.R., Simon, J.C., Girolomini, G., Tigelaar, R.E., and Cruz, P.D. jr. (1991) Sensitization and elicitation of inflammation in contact eczema. In: Czernielewski, J.M., Ed. Allergic Contact Dermatitis: Pathogenesis and Pharmacology. Basel: Karger. 58-65

Berke, G. (1994) The binding and lysis of target cells by cytotoxic lymphocytes: Molecular and cellular aspects. Ann. Rev. Immunol. *12*, 735-773

Berke, G. (1995). The CTL's kiss of death. Cell *81*, 9-12.

Bernhagen, J., Bacher, M., Calandra, T., Metz, C.N., Doty, S.B., Donnelly, T., and Bucala, R. (1996). An essential role for macrophage migration inhibitory factor in the tuberculin delayed-type hypersensitivity reaction. J. Exp. Med. *183*, 277-282.

Bianchi, A.T., Hooijkaas, H., Benner, R., Tees, R., Nordin, A.A., and Schreier, M.H. (1981). Clones of helper T cells mediate antigen-specific, H-2-restricted DTH. Nature *290*, 62-63.

Birkhofer, A., Rehbock, J., and Fricke, H. (1996). T lymphocytes from the normal human peritoneum contain high frequencies of Th2-type CD8+ T cells. Eur. J. Immunol. *26*, 957-960.

Bischoff, S.C., Brunner, T., De Weck, A.L., and Dahinden, C.A. (1990). Interleukin 5 modifies histamine release and leukotriene generation by human basophils in response to diverse agonists. J. Exp. Med. *172*, 1577-1582.

Blackman, M.J. and Morris, A.G. (1985). The effect of interferon treatment of targets on susceptibility to cytotoxic T-lymphocyte killing: augmentation of allogeneic killing and virus-specific killing relative to viral antigen expression. Immunol. *56*, 451-457.

Blazkovec, A.A., Sorkin, E., and Turk, J.L. (1965) A study of the passive cellular transfer of local cutaneous hypersensitivity, I, Passive transfer of delayed hypersensitivity in inbred and outbred guinea pigs. Int. Arch. Allergy *27*, 289

Bochner, B.S. and Schleimer, R.P. (1994). The role of adhesion molecules in human eosinophil and basophil recruitment. Journal of Allergy & Clinical Immunology *94*, 427-38; quiz 43.

Boey, H., Rosenbaum, R., Castracane, J., and Borish, L. (1989). Interleukin-4 is a neutrophil activator. Journal of Allergy & Clinical Immunology *83*, 978-984.

Bond, M.W., Shrader, B., Mosmann, T.R., and Coffman, R.L. (1987). A mouse T cell product that preferentially enhances IgA production. II. Physicochemical characterization. J. Immunol. *139*, 3691-3696.

Boughton, B. and Spector, W.G. (1963) Histology of the tuberculin reaction in guinea pigs. J. Path. Bact. *85*, 371

Bouley, D.M., Kanangat, S., Wire, W., and Rouse, B.T. (1995). Characterization of herpes simplex virus type-1 infection and herpetic stromal keratitis development in IFN-gamma knockout mice. J. Immunol. *155*, 3964-3971.

Bour, H., Peyron, E., Gaucherand, M., Garrigue, J.L., Desvignes, C., Kaiserlian, D., Revillard, J.P., and Nicolas, J.F. (1995). Major histocompatibility complex class I-restricted CD8+ T cells and class II-restricted CD4+ T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. Eur. J. Immunol. *25*, 3006-3010.

Briscoe, D.M., Cotran, R.S., and Pober, J.S. (1992). Effects of tumor necrosis factor, lipopolysaccharide, and IL-4 on the expression of vascular cell adhesion molecule-1 in vivo. Correlation with CD3+ T cell infiltration. J. Immunol. *149*, 2954-2960.

Brown, E.L., Rivas, J.M., Ullrich, S.E., Young, C.R., Norris, S.J., and Kripke, M.L. (1995). Modulation of immunity to Borrelia burgdorferi by ultraviolet irradiation: differential effect on Th1 and Th2 immune responses. Eur. J. Immunol. *25*, 3017-3022.

Buchanan, K.L. and Murphy, J.W. (1993). Characterization of cellular infiltrates and cytokine production during the expression phase of the anticryptococcal delayed-type hypersensitivity response. Infection & Immunity *61*, 2854-2865.

Buchanan, K.L. and Murphy, J.W. (1994). Regulation of cytokine production during the expression phase of the anticryptococcal delayed-type hypersensitivity response. Infection & Immunity *62*, 2930-2939.

Bucy, R.P., Panoskaltsis-Mortari, A., Huang, G.Q., Li, J., Karr, L., Ross, M., Russell, J.H., Murphy, K.M., and Weaver, C.T. (1994). Heterogeneity of single cell cytokine gene expression in clonal T cell populations. J. Exp. Med. *180*, 1251-1262.

Bukowski, J.F. and Welsh, R.M. (1985). Interferon enhances the susceptibility of virus-infected fibroblasts to cytotoxic T cells. J. Exp. Med. *161*, 257-262.

Callard, R.E. and Smith, S.H. (1988). Interleukin 2 and low molecular weight B cell growth factor are T cell-replacing factors for different subpopulations of human B cells. Eur. J. Immunol. *18*, 1635-1638.

Carter, L.L. and Dutton, R.W. (1995). Relative Perforin- and Fas-mediated lysis in T1 and T2 CD8 effector populations. J. Immunol. *155*, 1028-1031.

Chan, S.H., Perussia, B., Gupta, J.W., Kobayashi, M., Pospisil, M., Young, H.A., Wolf, S.F., Young, D., Clark, S.C., and Trinchieri, G. (1991). Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J. Exp. Med. *173*, 869-879.

Chang, C.H., Furue, M., and Tamaki, K. (1995). B7-1 expression of Langerhans cells is up-regulated by proinflammatory cytokines, and is down-regulated by interferon-gamma or by interleukin-10. Eur. J. Immunol. *25*, 394-398.

Chase, M.W. (1941) Inheritance in guinea pigs of the susceptibility to skin sensitization with simple chemical compounds. J. Exp. Med. *73*, 711

Chase, M.W. (1945) The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exp. Biol. Med. *59*, 134

Chase, M.W. (1954) Experimental sensitization with particular reference to picryl chloride. Int. Arch. Allergy *5*, 163

Chavance, M., Herbeth, B., and Lesourd, B. (1991). Delayed-type hypersensitivity and circulating basophils. A population study. Allergy *46*, 401-404.

Chensue, S.W., Ruth, J.H., Warmington, K., Lincoln, P. and Kunkel, S.L. (1995a) In vivo regulation of macrophage IL-12 production during type 1 and type 2 cytokine-mediated granuloma formation. J. Immunol. *155*, 3546-3551

Chensue, S.W., Warmington, K., Ruth, J., Lincoln, P., Kuo, M.C., and Kunkel, S.L. (1994). Cytokine responses during mycobacterial and schistosomal antigen-induced pulmonary granuloma formation. Production of Th1 and Th2 cytokines and relative contribution of tumor necrosis factor. American Journal of Pathology *145*, 1105-1113.

Chensue, S.W., Warmington, K.S., Ruth, J.H., Lincoln, P., and Kunkel, S.L. (1995b). Cytokine function during mycobacterial and schistosomal antigen-induced pulmonary granuloma formation. Local and regional participation of IFN-gamma, IL-10, and TNF. J. Immunol. *154*, 5969-5976.

Cher, D.J. and Mosmann, T.R. (1987). Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. J. Immunol. *138*, 3688-3694.

Chernoff, A.E., Granowitz, E.V., Shapiro, L., Vannier, E., Lonnemann, G., Angel, J.B., Kennedy, J.S., Rabson, A.R., Wolff, S.M., and Dinarello, C.A. (1995). A randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses. J. Immunol. *154*, 5492-5499.

Cherwinski, H.M., Schumacher, J.H., Brown, K.D., and Mosmann, T.R. (1987). Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J. Exp. Med. *166*, 1229-1244.

Chu, C.Q., Field, M., Andrew, E., Haskard, D., Feldmann, M., and Maini, R.N. (1992). Detection of cytokines at the site of tuberculin-induced delayed-type hypersensitivity in man. Clinical & Experimental Immunology *90*, 522-529.

Clerici, M. and Shearer, G.M. (1993). A TH1-->TH2 switch is a critical step in the etiology of HIV infection. Immunol. Today *14*, 107-111.

Coffman, R.L., Lee, F., Yokota, T., Arai, K., and Mosmann, T.R. (1987). The effect of BSF1 and IFNg upon mouse immunoglobulin isotype expression. In UCLA Symposium: Immune regulation by characterized polypeptides. G. Goldstein, J. Bach, and H. Wigzell, eds. (New York: Alan Liss), pp. 523-532.

Coffman, R.L., Seymour, B.W., Lebman, D.A., Hiraki, D.D., Christiansen, J.A., Shrader, B., Cherwinski, H.M., Savelkoul, H.F., Finkelman, F.D., Bond, M.W., and

Mosmann, T.R. (1988). The role of helper T cell products in mouse B cell differentiation and isotype regulation. Immunol. Rev. *102*, 5-28.

Cohen, S., Pick, E. and Oppenheim, J.J. (1979) In Biology of Lymphokines, Pick, E. and Oppenheim, J.J. (Eds). New York: Academic Press, pp 1-7

Colditz, I.G. and Watson, D.L. (1992). The effect of cytokines and chemotactic agonists on the migration of T lymphocytes into skin. Immunol. *76*, 272-278.

Corcoran, M.L., Stetler-Stevenson, W.G., Brown, P.D., and Wahl, L.M. (1992). Interleukin 4 inhibition of prostaglandin E2 synthesis blocks interstitial collagenase and 92-kDa type IV collagenase/gelatinase production by human monocytes. Journal of Biological Chemistry *267*, 515-519.

Coyle, A.J., Erard, F., Bertrand, C., Walti, S., Pircher, H., and Le Gros, G. (1995). Virus-specific CD8+ cells can switch to Interleukin 5 production and induce airway eosinophilia. J. Exp. Med. *181*, 1229-1233.

Cramer, D.V. (1987). Cardiac transplantation: immune mechanisms and alloantigens involved in graft rejection. Critical Reviews in Immunology *7*, 1-30.

Crawford, R., Finbloom, M., Ohara, J., Paul, W.E., and Meltzer, M. (1988). B cell stimulatory factor 1 (interleukin 4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. J. Immunol. *139*, 135-141.

Croft, M., Carter, L., Swain, S.L., and Dutton, R.W. (1994). Generation of polarized antigen-specific CD8 effector populations: Reciprocal action of interleukin (IL)-4 and IL-12 in promoting Type 2 versus Type 1 cytokine profiles. J. Exp. Med. *180*, 1715-1728.

Crowle, A.J. (1975). Delayed hypersensitivity in the mouse. Adv. Immunol. *20*, 197-264.

Cruz, P.D. Jr. (1994) What accounts for contact allergy? Am J Contact Dermatitis. *4*, 189

Cunha, F.Q., Moncada, S., and Liew, F.Y. (1992). Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-gamma in murine macrophages. Biochemical and Biophysical Research Communications *182*, 1155-1159.

Darmon, A.J., Nicholson, D.W. and Bleackley, R.C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. Nature, *377*, 446-448

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., and de Vries, J.E. (1991a). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. *174*, 1209-1220.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M.G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H., and de Vries, J.E. (1991b). Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J. Exp. Med. *174*, 915-924.

Defrance, T., Vanbervliet, B., Briere, F., Durand, I., Rousset, F., and Banchereau, J. (1992). Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. J. Exp. Med. *175*, 671-682.

Del Prete, G., Maggi, E., and Romagnani, S. (1994). Human Th1 and Th2 cells: functional properties, mechanisms of regulation, and role in disease. Laboratory Investigation *70*, 299-306.

Del Prete, G., Maggi, E., Pizzolo, G., and Romagnani, S. (1995). CD30, Th2 cytokines and HIV infection: a complex and fascinating link. Immunol. Today *16*, 76-80.

Del Prete, G., Ricci, M., and Romagnani, S. (1991a). T cells, IgE antibodies, cytokines and allergic inflammation. Allergie et Immunologie *23*, 239-243.

Del Prete, G.F., De Carli, M., Mastromauro, C., Biagiotti, R., Macchia, D., Falagiani, P., Ricci, M., and Romagnani, S. (1991b). Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. J. Clin. Invest. *88*, 346-350.

Del Prete, G.F., De Carli, M., Ricci, M., and Romagnani, S. (1991c). Helper activity for immunoglobulin synthesis of T helper type 1 (Th1) and Th2 human T cell clones: the help of Th1 clones is limited by their cytolytic capacity. J. Exp. Med. *174*, 809-813.

Denburg, J.A., Silver, J.E., and Abrams, J.S. (1991). Interleukin-5 is a human basophilopoietin: induction of histamine content and basophilic differentiation of HL-60 cells and of peripheral blood basophil-eosinophil progenitors. Blood *77*, 1462-1468.

Denkins, Y., Fidler, I.J., and Kripke, M.L. (1989). Exposure of mice to UV-B radiation suppresses delayed hypersensitivity to Candida albicans. Photochemistry & Photobiology *49*, 615-619.

Dent, L.A., Strath, M., Mellor, A.L., and Sanderson, C.J. (1990). Eosinophilia in transgenic mice expressing interleukin 5. J. Exp. Med. *172*, 1425-1431.

Devergne, O., Marfaing-Koka, A., Schall, T.J., Leger-Ravet, M.B., Sadick, M., Peuchmaur, M., Crevon, M.C., Kim, K.J., Schall, T.T., Kim, T., and et al, (1994). Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells [published erratum appears in J Exp Med 1994 Aug 1;180(2):775]. J. Exp. Med. *179*, 1689-1694.

Dieli, F., Asherson, G.L., Romano, G.C., Sireci, G., Gervasi, F., and Salerno, A. (1994). IL-4 is essential for the systemic transfer of delayed hypersensitivity by T cell lines. Role of gamma/delta cells. J. Immunol. *152*, 2698-2704.

Ding, L. and Shevach, E.M. (1992). IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. J. Immunol. *148*, 3133-3139.

Ding, L., Linsley, P.S., Huang, L.-Y., Germain, R.N., and Shevach, E.M. (1993). IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. J. Immunol. *151*, 1224-1234.

Doherty, M.L., Bassett, H.F., Quinn, P.J., Davis, W.C., Kelly, A.P. and Monaghan, M.L. (1996) A sequential study of the bovine tuberculin reaction. Immunology *87*, 9

Doherty, P.C., Allan, J.E., Lynch, F., and Ceredig, R. (1990). Dissection of an inflammatory process induced by CD8+ T cells. Immunol. Today *11*, 55-59.

Dorf, M.E. and Benacerraf, B. (1984). Suppressor cells and immunoregulation. Ann. Rev. Immunol. *2*, 127-157.

Dresser, D.W. (1991). The potentiating effect of pregnancy on humoral immune responses of mice. J. Reprod. Immunol. *20*, 253-266.

Dubois, G.R., Bruijnzeel-Koomen, C.A., and Bruijnzeel, P.L. (1994). IL-4 induces chemotaxis of blood eosinophils from atopic dermatitis patients, but not from normal individuals. J. Invest. Dermatol. *102*, 843-846.

Dvorak, H.F., Dvorak, A.M., Simpson, B.A., Richerson, H.B., Leskowitz, S., and Karnovsky, M.J. (1970). Cutaneous basophil hypersensitivity. II. A light and electron microscopic description. J. Exp. Med. *132*, 558-582.

Dvorak, H.F., Galli, S.J., and Dvorak, A.M. (1980). Expression of cell-mediated hypersensitivity in vivo - recent advances. Int. Rev. Exp. Pathol. *21*, 119-194.

Eisen, H.N. and Tabachnick, M. (1958) Elicitation of allergic contact dermatitis in the guinea pig: The distribution of bound dinitrobenzene groups within the skin and quantitative determination of the extent of combination of 2,4-dinitrochlorobenzene with epidermal protein in vivo. J. Exp. Med. *108*, 773

Eisen, H.N., Orris, L. and Belman, S. (1952) Elicitation of delayed allergic skin reaction with haptens: the dependence of elicitation of hapten combination with protein. J. Exp. Med *95*, 473

Elices, M.J., Tamraz, S., Tollefson, V., and Vollger, L.W. (1993). The integrin VLA-4 mediates leukocyte recruitment to skin inflammatory sites in vivo. Clinical & Experimental Rheumatology *11 Suppl 8*, S77-S80.

Else, K.J., Finkelman, F.D., Maliszewski, C.R., and Grencis, R.K. (1994). Cytokine-mediated regulation of chronic intestinal helminth infection. J. Exp. Med. *179*, 347-351.

Erard, F., Corthesy, P., Nabholz, M., Lowenthal, J.W., Zaech, P., Plaetinck, G., and MacDonald, H.R. (1985). Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. J. Immunol. *134*, 1644-1652.

Essner, R., Rhoades, K., McBride, W.H., Morton, D.L., and Economou, J.S. (1989). IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. J. Immunol. *142*, 3857-3861.

Favre, C., Saeland, S., Caux, C., Duvert, V., and de Vries, J.E. (1990). Interleukin-4 has basophilic and eosinophilic cell growth-promoting activity on cord blood cells. Blood *75*, 67-73.

Ferguson, T.A., Dube, P., and Griffith, T.S. (1994). Regulation of Contact Hypersensitivity by Interleukin 10. J. Exp. Med. *179*, 1597-1604.

Fernandez-Botran, R., Sanders, V.M., Mosmann, T.R., and Vitetta, E.S. (1988). Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. J. Exp. Med. *168*, 543-558.

Finkelman, F.D., Pearce, E.J., Urban, J.F.,Jr., and Sher, A. (1991). Regulation and biological function of helminth-induced cytokine responses. Immunol. Today *12*, A62-A66.

Fiorentino, D.F., Bond, M.W., and Mosmann, T.R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. *170*, 2081-2095.

Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., and O'Garra, A.O. (1991a). IL10 inhibits cytokine production by activated macrophages. J. Immunol. *147*, 3815-3822.

Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W., and O'Garra, A. (1991b). IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J. Immunol. *146*, 3444-3451.

Flax, M.H. and Caulfield, J.B. (1963) Cellular and vascular components of allergic contact dermatitis, light and electron microscopic observations. Amer. J. Path. *43*, 1031

Flores Villanueva, P.O., Reiser, H., and Stadecker, M.J. (1994). Regulation of T helper cell responses in experimental murine schistosomiasis by IL-10. Effect on expression of B7 and B7-2 costimulatory molecules by macrophages. J. Immunol. *153*, 5190-5199.

Fong, T.A. and Mosmann, T.R. (1989). The role of IFN-gamma in delayed-type hypersensitivity mediated by Th1 clones. J. Immunol. *143*, 2887-2893.

Fong, T.A. and Mosmann, T.R. (1990). Alloreactive murine CD8+ T cell clones secrete the Th1 pattern of cytokines. J. Immunol. *144*, 1744-1752.

Frankenberger, M., Pechumer, H., and Ziegler-Heitbrock, H.W. (1995). Interleukin-10 is upregulated in LPS tolerance. Journal of Inflammation *45*, 56-63.

Gaga, M., Frew, A.J., Varney, V.A., and Kay, A.B. (1991). Eosinophil activation and T lymphocyte infiltration in allergen-induced late phase skin reactions and classical delayed-type hypersensitivity. J. Immunol. *147*, 816-822.

Gajewski, T.F. and Fitch, F.W. (1988). Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J. Immunol. *140*, 4245-4252.

Gauchat, J.F., Aversa, G., Gascan, H., and de Vries, J.E. (1992a). Modulation of IL-4 induced germline epsilon RNA synthesis in human B cells by tumor necrosis factor-alpha, anti-CD40 monoclonal antibodies or transforming growth factor-beta correlates with levels of IgE production. Int. Immunol. *4*, 397-406.

Gauchat, J.F., Gascan, H., de Waal Malefyt, R., and de Vries, J.E. (1992b). Regulation of germ-line epsilon transcription and induction of epsilon switching in cloned EBV-transformed and malignant human B cell lines by cytokines and CD4+ T cells. J. Immunol. *148*, 2291-2299.

Gautam, S., Battisto, J., Major, J.A., Armstrong, D., Stoler, M., and Hamilton, T.A. (1994). Chemokine expression in trinitrochlorobenzene-mediated contact hypersensitivity. Journal of Leukocyte Biology *55*, 452-460.

Gazzinelli, R.T., Makino, M., Chattopadhyay, S.K., Snapper, C.M., Sher, A., Hugin, A.W., and Morse, H.C. (1992a). CD4+ subset regulation in viral infection. Preferential activation of Th2 cells during progression of retrovirus-induced immunodeficiency in mice. J. Immunol. *148*, 182-188.

Gazzinelli, R.T., Oswald, I.P., Hieny, S., James, S.L., and Sher, A. (1992b). The microbicidal activity of interferon-gamma-treated macrophages against Trypanosoma cruzi involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-beta. Eur. J. Immunol. *22*, 2501-2506.

Gazzinelli, R.T., Oswald, I.P., James, S.L., and Sher, A. (1992c). IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages. J. Immunol. *148*, 1792-1796.

Geczy, C.L. (1984). The role of lymphokines in delayed-type hypersensitivity reactions. Springer Seminars in Immunopathology *7*, 321-346.

Gemmell, E. and Seymour, G.J. (1994). Cytokines and T cell switching. Critical Reviews in Oral Biology & Medicine *5*, 249-279.

Ghildyal, N., McNeil, H.P., Gurish, M.F., Austen, K.F., and Stevens, R.L. (1992a). Transcriptional regulation of the mucosal mast cell-specific protease gene, MMCP-2, by interleukin 10 and interleukin 3. J. Biol. Chem. *267*, 8473-8477.

Ghildyal, N., McNeil, H.P., Stechschulte, S., Austen, K.F., Silberstein, D., Gurish, M.F., Somerville, L.L., and Stevens, R.L. (1992b). IL-10 induces transcription of the gene for mouse mast cell protease-1, a serine protease preferentially expressed in mucosal mast cells of Trichinella spiralis-infected mice. J. Immunol. *149*, 2123-2129.

Glimcher, L.H., Sharrow, S.O., and Paul, W.E. (1983). Serologic and functional characterization of a panel of antigen-presenting cell lines expressing mutant I-A class II molecules. J. Exp. Med. *158*, 1573-1588.

Gocinski, B.L. and Tigelaar, R.E. (1990). Roles of CD4+ and CD8+ T cells in murine contact sensitivity revealed by in vivo monoclonal antibody depletion. J. Immunol. *144*, 4121-4128.

Golding, H. and Singer, A. (1985). Specificity, phenotype, and precursor frequency of primary cytolytic T lymphocytes specific for class II major histocompatibility antigens. J. Immunol. *135*, 1610-1615.

Goossens, P.L., Marchal, G., and Milon, G. (1992). Transfer of both protection and delayed-type hypersensitivity against live Listeria is mediated by the CD8+ T cell subset: a study with Listeria-specific T lymphocytes recovered from murine infected liver. Int. Immunol. *4*, 591-598.

Gordon, C. and Wofsy, D. (1990). Effects of recombinant murine tumor necrosis factor-alpha on immune function. J. Immunol. *144*, 1753-1758.

Gordon, J., Millsum, M.J., Guy, G.R., and Ledbetter, J.A. (1988). Resting B lymphocytes can be triggered directly through the CDw40 (Bp50) antigen. A comparison with IL-4-mediated signaling. J. Immunol. *140*, 1425-1430.

Haanen, J.B., de Waal Malefijt, R., Res, P.C., Kraakman, E.M., Ottenhoff, T.H., de Vries, R.R., and Spits, H. (1991). Selection of a human T helper type 1-like T cell subset by mycobacteria. J. Exp. Med. *174*, 583-592.

Hamaguchi, Y., Kanakura, Y., Fujita, J., Takeda, S., Nakano, T., Tarui, S., Honjo, T., and Kitamura, Y. (1987). Interleukin 4 as an essential factor for in vitro clonal growth of murine connective tissue-type mast cells. J. Exp. Med. *165*, 268-273.

Hamid, Q., Azzawi, M., Ying, S., Moqbel, R., Wardlaw, A.J., Corrigan, C.J., Bradley, B., Durhan, S.R., Collins, J.V., Jeffery, P.K., Quint, D.J. and Kay, A. (1991) Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma, J. Clin. Invest. 87: 1541-46

Hamilton, T.A. and Adams, D.O. (1987) Molecular mechanisms of signal transduction in macrophages. Immunol. Today *8*, 151-158.

Harada, M., Mori, K., and Nishimoto, H. (1986). Suppressive and augmentative effects of recombinant human interleukin-2 upon delayed type of hypersensitivity in the mouse. Inflammation *10*, 167-174.

Hart, P.H., Ahern, M.J., Smith, M.D., and Finlay-Jones, J.J. (1995). Comparison of the suppressive effects of interleukin-10 and interleukin-4 on synovial fluid macrophages and blood monocytes from patients with inflammatory arthritis. Immunol. *84*, 536-542.

Hart, P.H., Vitti, G.F., Burgess, D.R., Whitty, G.A., Piccoli, D.S., and Hamilton, J.A. (1989). Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. Proc. Natl. Acad. Sci. USA *86*, 3803-3807.

Heinzel, F.P., Sadick, M.D., Holaday, B.J., Coffman, R.L., and Locksley, R.M. (1989). Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. J. Exp. Med. *169*, 59-72.

Heinzel, F.P., Sadick, M.D., Mutha, S.S., and Locksley, R.M. (1991). Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis. Proc. Natl. Acad. Sci. USA *88*, 7011-7015.

Henkart, P.A. (1994). Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. Immunity *1*, 343-346.

Hernandez-Pando, R. and Rook, G.A. (1994). The role of TNF-alpha in T-cell-mediated inflammation depends on the Th1/Th2 cytokine balance. Immunol. *82*, 591-595.

Hernandez-Pando, R., Orozco, H., and Mancilla, R. (1995). T-cell lung granulomas induced by sepharose-coupled Mycobacterium tuberculosis protein antigens: immunosuppressive phenomena reversed with cyclophosphamide and indomethacin. Immunol. *86*, 506-511.

Herrmann, P., Schreier, M.H., Borel, J.F., and Feurer, C. (1988). Mast cell degranulation as a major event in the effector phase of delayed-type hypersensitivity induced by cloned helper T cells. International Archives of Allergy & Applied Immunology *86*, 102-105.

Hestdal, K., Ruscetti, F.W., Ihle, J.N., Jacobsen, S.E., Dubois, C.M., Kopp, W.C., Longo, D.L., and Keller, J.R. (1991). Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. J. Immunol. *147*, 22-28.

Higashi, N., Yoshizuka, N., Ohuchi, A., Osawa, T., and Kobayashi, Y. (1995). Involvement of inflammatory cytokines in a delayed-type hypersensitivity reaction. Cell. Immunol. *161*, 288-294.

Hill, J.O., Awwad, M., and North, R.J. (1989). Elimination of CD4+ suppressor T cells from susceptible BALB/c mice releases CD8+ T lymphocytes to mediate protective immunity against Leishmania. J. Exp. Med. *169*, 1819-1827.

Ho, J.L., He, S.H., Rios, M.J., and Wick, E.A. (1992). Interleukin-4 inhibits human macrophage activation by tumor necrosis factor, granulocyte-monocyte colony-stimulating factor, and interleukin-3 for antileishmanial activity and oxidative burst capacity. J. Infect. Dis. *165*, 344-351.

Ho, M.K. and Springer, T.A. (1983). Tissue distribution, structural characterization, and biosynthesis of Mac-3, a macrophage surface glycoprotein exhibiting molecular weight heterogeneity. Journal of Biological Chemistry *258*, 636-642.

Hogg, N. (1991) Roll, roll, roll your leukocyte gently down the vein.... Immunol. Today *13*, 113-115.

Holaday, B.J., Sadick, M.D., Wang, Z.E., Reiner, S.L., Heinzel, F.P., Parslow, T.G., and Locksley, R.M. (1991). Reconstitution of Leishmania immunity in severe combined immunodeficient mice using Th1- and Th2-like cell lines. J. Immunol. *147*, 1653-1658.

Holland, D., Bretscher, P., and Russell, A.S. (1984). Immunologic and inflammatory responses during pregnancy. J. Clin. Lab. Immunol. *14*, 177-179.

Hosken, N.A., Shibuya, K., Heath, A.W., Murphy, K.M., and O'Garra, A. (1995). The effect of antigen dose on CD4+ T helper cell phenotype development in an abTCR-transgenic model. J. Exp. Med. *182*, 1579-1584.

Howard, J.G., Hale, C., and Liew, F.Y. (1981). Immunological regulation of experimental cutaneous leishmaniasis. IV. Prophylactic effect of sublethal irradiation as a result of abrogation of suppressor T cell generation in mice genetically susceptible to Leishmania tropica. J. Exp. Med. *153*, 557-568.

Howard, M., Muchamuel, T., Andrade, S., and Menon, S. (1993). Interleukin 10 protects mice from lethal endotoxemia. J. Exp. Med. *177*, 1205-1208.

Hsieh, C.-S., Macatonia, S.E., O'Garra, A., and Murphy, K.M. (1993). Pathogen-induced Th1 phenotype development in CD4+ alpha beta-TCR transgenic T cells is macrophage dependent. Int. Immunol. *5*, 371-382.

Hsu, D.H., Moore, K.W., and Spits, H. (1992). Differential effects of IL-4 and IL-10 on IL-2-induced IFN-gamma synthesis and lymphokine-activated killer activity. Int. Immunol. *4*, 563-569.

Huhn, R.D., Radwanski, E., O'Connell, S.M., Sturgill, M.G., Clarke, L., Cody, R.P., Affrime, M.B., and Cutler, D.L. (1996). Pharmacokinetics and immunomodulatory properties of intravenously administered recombinant human interleukin-10 in healthy volunteers. Blood *87*, 699-705.

Issekutz, T.B. (1991). Inhibition of in vivo lymphocyte migration to inflammation and homing to lymphoid tissues by the TA-2 monoclonal antibody. A likely role for VLA-4 in vivo. J. Immunol. *147*, 4178-4184.

Issekutz, T.B., Stoltz, J.M., and v.d.Meide, P. (1988a). Lymphocyte recruitment in delayed-type hypersensitivity. The role of IFN-gamma. J. Immunol. *140*, 2989-2993.

Issekutz, T.B., Stoltz, J.M., and van der Meide, P. (1988b). The recruitment of lymphocytes into the skin by T cell lymphokines: the role of gamma-interferon. Clinical & Experimental Immunology *73*, 70-75.

Jayaraman, S., Martin, C.A., and Dorf, M.E. (1990). Enhancement of in vivo cell-mediated immune responses by three distinct cytokines. J. Immunol. *144*, 942-951.

Jinquan, T., Larsen, C.G., Gesser, B., Matsushima, K., and Thestrup-Pedersen, K. (1993). Human IL-10 is a chemoattractant for CD8+ T lymphocytes and an inhibitor of IL-8-induced CD4+ T lymphocyte migration. J. Immunol. *151*, 4545-4551.

Jones, T.D. and Mote, J.R. (1934) The phases of foreign protein sensitization in human beings. New Engl. J. Med. *210*, 120

Ju, S.T. (1991). Distinct pathways of CD4 and CD8 cells induce rapid target DNA fragmentation. J. Immunol. *146*, 812-818.

Kagi, D., Ledermann, B., Burki, K., Hengartner, H. and Zinkernagel, R.M. (1994c) CD8 + T cell mediated protection against an intracellular bacterium by perforin dependent cytotoxicity. Eur. J. Immunol. *24*, 3068-3072.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994a). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31-37.

Kagi, D., Odermatt, B., Ohashi, P.S., Zinkernagel, R.M., and Hengartner, H. (1996). Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. J. Exp. Med. *183*, 2143-2152.

Kagi, D., Seiler, P., Pavlovic, J., Ledermann, B., Burki, K., Zinkernagel, R.M., and Hengartner, H. (1995). The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. Eur. J. Immunol. *25*, 3256-3262.

Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994b). Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science *265*, 528-530.

Kalaaji, A.N., McCullough, K., and Hay, J.B. (1989). The enhancement of lymphocyte localization in skin sites of sheep by tumor necrosis factor alpha. Immunol. Lett. *23*, 143-147.

Kalish, R.S. (1991). Recent developments in the pathogenesis of allergic contact dermatitis. Archives of Dermatology *127*, 1558-1563.

Kamogawa, Y., Minasi, L.A., Carding, S.R., Bottomly, K., and Flavell, R.A. (1993). The relationship of IL-4- and IFN gamma-producing T cells studied by lineage ablation of IL-4-producing cells. Cell *75*, 985-995.

Kapiotis, S., Besemer, J., Bevec, D., Valent, P., Bettelheim, P., Lechner, K., and Speiser, W. (1991). Interleukin-4 counteracts pyrogen-induced downregulation of thrombomodulin in cultured human vascular endothelial cells. Blood *78*, 410-415.

Kaplan, G., Luster, A.D., Hancock, G., and Cohn, Z.A. (1987). The expression of a gamma interferon-induced protein (IP-10) in delayed immune responses in human skin. J. Exp. Med. *166*, 1098-1108.

Kapsenberg, M.L., Wierenga, E.A., Stiekema, F.E., Tiggelman, A.M., and Bos, J.D. (1992). Th1 lymphokine production profiles of nickel-specific CD4+T-lymphocyte clones from nickel contact allergic and non-allergic individuals. J. Invest. Dermatol. *98*, 59-63.

Kasama, T., Strieter, R.M., Lukacs, N.W., Burdick, M.D., and Kunkel, S.L. (1994). Regulation of neutrophil-derived chemokine expression by IL-10. J. Immunol. *152*, 3559-3569.

Katsura, Y. (1977). Cell-mediated and humoral immune responses in mice. III. Dynamic balance between delayed-type hypersensitivity and antibody response. Immunol. *32*, 227-235.

Kawamura, T. and Furue, M. (1995). Comparative analysis of B7-1 and B7-2 expression in Langerhans cells: differential regulation by T helper type 1 and T helper type 2 cytokines. Eur. J. Immunol. *25*, 1913-1917.

Keane-Myers, A. and Nickell, S.P. (1995). Role of IL-4 and IFN-gamma in modulation of immunity to Borrelia burgdorferi in mice. J. Immunol. *155*, 2020-2028.

Kelley, K.A. and Pitha, P.M. (1985). Characterization of a mouse interferon gene locus II. Differential expression of alpha-interferon genes. Nucleic Acids Research *13*, 825-839.

Kelso, A. (1995). Th1 and Th2 subsets: paradigms lost? Immunol. Today *16*, 374-379.

Kirchheimer, W.F., Weiser, R.S., and Liew, R.Van. (1949) The tuberculin reaction. III. Transfer of systemic tuberculin sensitivity with cells of tuberculous guinea pigs. Proc, Soc. Exp. Biol. Med. *70*, 99

Klasen, I.S., van der Kwast, T.H., Donselaar, I.G., Ladestein, R.M., and Benner, R. (1987). Flare-up of delayed-type hypersensitivity initially induced by murine cloned helper T cells. Cell. Immunol. *108*, 235-241.

Klein, N.J., Rigley, K.P., and Callard, R.E. (1993). IL-4 regulates the morphology, cytoskeleton, and proliferation of human umbilical vein endothelial cells: relationship between vimentin and CD23. Int. Immunol. *5*, 293-301.

Kobayashi, M., Fitz, L., Ryan, M., Hewick, R.M., Clark, S.C., Chan, S., Loudon, R., Sherman, F., Perussia, B., and Trinchieri, G. (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J. Exp. Med. *170*, 827-845.

Koch, R. (1890) Weitere Mitteilungen ueber ein Heilmittel gegen Tuberkulose. Dtsch. med. Wschr. *16*, 1029

Kondo, S., McKenzie, R.C., and Sauder, D.N. (1994). Interleukin-10 inhibits the elicitation phase of allergic contact hypersensitivity. J. Invest. Dermatol. *103*, 811-814.

Krakauer, T. (1995). IL-10 inhibits the adhesion of leukocytic cells to IL-1-activated human endothelial cells. Immunol. Lett. *45*, 61-65.

Krammer, P.H., Dhein, J., Walczak, H., Behrmann, I., Mariani, S., Matiba, B., Fath, M., Daniel, P.T., Knipping, E., Westendorp, M.O., Stricker, K., Baumler, C., Hellbardt, S., Germer, M., Peter, M.E. and Debatin, K. (1994) The role of APO-1-mediated apoptosis in the immune system. Immunol. rev. *142*, 175-191

Krensky, A.M., Reiss, C.S., Mier, J.W., Strominger, J.L., and Burakoff, S.J. (1982). Long-term human cytolytic T-cell lines allospecific for HLA-DR6 antigen are OKT4+. Proc. Natl. Acad. Sci. USA *79*, 2365-2369.

Krishnan, L., Guilbert, L.J., Russell, A.S., Wegmann, T.G., Mosmann, T.R., and Belosevic, M. (1996). Pregnancy impairs resistance of C57Bl/6 mice to *Leishmania major* infection and causes decreased antigen-specific IFNg responses and increased production of TH2 cytokines. J. Immunol. *156*, 644-652.

Kucharzik, T., Stoll, R., Lugering, N., and Domschke, W. (1995). Circulating antiinflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). Clinical & Experimental Immunology *100*, 452-456.

Kuchroo, V.K., Steele, J.K., O'Hara, R.M.,Jr., Jayaraman, S., Selvaraj, P., Greenfield, E., Kubo, R.T., and Dorf, M.E. (1990). Relationships between antigen-specific helper and inducer suppressor T cell hybridomas. J. Immunol. *145*, 438-448.

Kudo, C., Yamashita, T., Araki, A., Terashita, M. Watananbe, T., Atsumi, M., Tamura, M. and Sendo, F. (1993a) Modulation of in vivo immune response by selective depletion of neutrophils using a monoclonal antibody, RP-3. I. Inhibition by RP-3 treatment of the priming and effector phases of delayed-type hypersensitivity to sheep red blood cells in rats. J. Immunol. *150*, 3728-3738.

Kudo, C., Yamashita, T., Terashita, M. and Sendo, F. (1993b) Modulation of in vivo immune response by selective depletion of neutrophils using a monoclonal antibody, RP-3. II. Inhibition by RP-3 treatment of mononuclear leukocyte recruitment in delayed-type hypersensitivity to sheep red blood cells in rats. J. Immunol. *150*, 3739-3746.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 1-20.

Kuhn, R., Rajewsky, K., and Muller, W. (1991). Generation and analysis of interleukin-4 deficient mice. Science *254*, 707-710.

Kullberg, M.C., Pearce, E.J., Hieny, S.E., Sher, A., and Berzofsky, J.A. (1992). Infection with Schistosoma mansoni alters Th1/Th2 cytokine responses to a non-parasite antigen. J. Immunol. *148*, 3264-3270.

Kundig, T.M., Althage, A., Hengartner, H., and Zinkernagel, R.M. (1992). Skin test to assess virus-specific cytotoxic T-cell activity. Proc. Natl. Acad. Sci. USA *89*, 7757-7761.

Lacraz, S., Nicod, L., Galve-de Rochemonteix, B., Baumberger, C., Dayer, J.M., and Welgus, H.G. (1992). Suppression of metalloproteinase biosynthesis in human alveolar macrophages by interleukin-4. J. Clin. Invest. *90*, 382-388.

Lancki, D.W., Hsieh, C.-S., and Fitch, F.W. (1991). Mechanisms of lysis by cytotoxic T lymphocyte clones. Lytic activity and gene expression in cloned antigen-specific CD4+ and CD8+ T lymphocytes. J. Immunol. *146*, 3242-3249.

Landsteiner, K. and Jacobs, J.L. (1936) Studies on the sensitization of animals with simple chemical compounds. II. J. Exp. Med. *64*, 625

Larsen, C.G., Thomsen, M.K., Gesser, B., Thomsen, P.D., Deleuran, B.W., Nowak, J., Skodt, V., Thomsen, H.K., Deleuran, M., Thestrup-Pedersen, K., and et al, (1995). The delayed-type hypersensitivity reaction is dependent on IL-8. Inhibition of a tuberculin skin reaction by an anti-IL-8 monoclonal antibody. J. Immunol. *155*, 2151-2157.

Le, J., Prensky, W., Yip, Y.K., Chang, Z., Hoffman, T., Stevenson, H.C., Balazs, I., Sadlik, J.R., and Vilcek, J. (1983). Activation of human monocyte cytotoxicity by natural and recombinant immune interferon. J. Immunol. *131*, 2821-2826.

Lebman, D.A. and Coffman, R.L. (1988a). Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. J. Exp. Med. *168*, 853-862.

Lebman, D.A. and Coffman, R.L. (1988b). The effects of IL-4 and IL-5 on the IgA response by murine Peyer's patch B cell subpopulations. J. Immunol. *141*, 2050-2056.

Lebman, D.A., Nomura, D.Y., Coffman, R.L., and Lee, F.D. (1990). Molecular Characterization of Germ-Line Immunoglobulin-A Transcripts Produced During Transforming Growth Factor Type Beta-Induced Isotype Switching. Proc. Natl. Acad. Sci. USA *87*, 3962-3966.

Lee, D.G., Cho, B.H., Hervey, J., and Daynes, R.A. (1988). The role of neutrophils in tissue localized cell-mediated immunologic responses: I. The intensity of contact-type and delayed-type hypersensitivity responses may be influenced by the extent of extracellular matrix degradation by neutrophil proteases. Regional Immunology *1*, 149-162.

Lee, F., Yokota, T., Otsuka, T., Meyerson, P., Villaret, D., Coffman, R., Mosmann, T.R., Rennick, D., Roehm, N., Smith, C., Zlotnik, A., and Arai, K. (1986). Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell- and mast-cell-stimulating activities. Proc. Natl. Acad. Sci. USA *83*, 2061-2065.

Lee, S.H., Aggarwal, B.B., Rinderknecht, E., Assisi, F., and Chiu, H. (1984). The synergistic anti-proliferative effect of gamma-interferon and human lymphotoxin. J. Immunol. *133*, 1083-1086.

Leung, K.N. and Ada, G.L. (1980). Two T-cell populations mediate delayed-type hypersensitivity to murine influenza virus infection. Scandinavian Journal of Immunology *12*, 481-487.

Lewis, D.B., Larsen, A., and Wilson, C.B. (1986). Reduced interferon-gamma mRNA levels in human neonates. Evidence for an intrinsic T cell deficiency independent of other genes involved in T. J. Exp. Med. *163*, 1018-1023.

Li, L., Elliott, J.F., and Mosmann, T.R. (1994). IL10 Inhibits Cytokine Production, Vascular Leakage And Swelling During TH1-Induced Delayed Type Hypersensitivity. J. Immunol. *153*, 3967-3978.

Liew, F.Y. (1982). Regulation of Delayed Type Hypersensitivity to pathogens and alloantigens. Immunol. Today *3*, 18-23.

Liew, F.Y. (1983). Specific suppression of responses to Leishmania tropica by a cloned T-cell line. Nature *305*, 630-632.

Lin, H., Mosmann, T.R., Guilbert, L., Tuntipopipat, S., and Wegmann, T.G. (1993). Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J. Immunol. *151*, 4562-4573.

Lin, Y.L. and Askonas, B.A. (1981). Biological properties of an influenza A virus-specific killer T cell clone. Inhibition of virus replication in vivo and induction of delayed-type hypersensitivity reactions. J. Exp. Med. *154*, 225-234.

Liu, C.C., Walsh, C.M., and Young, J.D. (1995). Perforin: structure and function. Immunol. Today *16*, 194-201.

Locksley, R.M. (1994). Th2 cells: help for helminths. J. Exp. Med. *179*, 1405-1407.

Locksley, R.M. and Louis, J.A. (1992). Immunology of leishmaniasis. Curr. Opin. Immunol. *4*, 413-418.

Locksley, R.M. and Scott, P. (1991). Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. Immunol. Today *12*, A58-A61.

Lopez, A.F., Begley, C.G., Williamson, D.J., Warren, D.J., Vadas, M.A., and Sanderson, C.J. (1986). Murine eosinophil differentiation factor. An eosinophil-specific colony-stimulating factor with activity for human cells. J. Exp. Med. *163*, 1085-1099.

Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J. (1994). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature *370*, 650-652.

Lu, X., Ledermann, B., and Borel, J.F. (1995). Survival of islet grafts in perforin-deficient mice. Transplant. Proc. *27*, 3262-3263.

Macatonia, S.E., Doherty, T.M., Knight, S.C., and O'Garra, A. (1993). Differential effect of IL-10 on dendritic cell-induced T cell proliferation and IFN-gamma production. J. Immunol. *150*, 3755-3765.

MacPhee, M.J., Gordon, J., Christou, N.V., Sanchez-Cantu, L., and Rode, H.H. (1993). Cells recovered from human DTH reactions: phenotypic and functional analysis. Cell. Immunol. *151*, 80-96.

Maggi, E., Giudizi, M.G., Biagiotti, R., Annunziato, F., Manetti, R., Piccinni, M.P., Parronchi, P., Sampognaro, S., Giannarini, L., Zuccati, G., and Romagnani, S. (1994).

Th2-like CD8+ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. J. Exp. Med. *180*, 489-495.

Man, S., Lechler, R.I., Batchelor, J.R., and Sharrock, C.E. (1990). Individual variation in the frequency of HLA class II-specific cytotoxic T lymphocyte precursors. Eur. J. Immunol. *20*, 847-854.

Marchal, G., Seman, M., Milon, G., Truffa-Bachi, P., and Zilberfarb, V. (1982). Local adoptive transfer of skin delayed type hypersensitivity initiated by a single T lymphocyte. J. Immunol. *129*, 954-958.

Marfaing-Koka, A., Devergne, O., Gorgone, G., Portier, A., Schall, T.J., Galanaud, P., and Emilie, D. (1995). Regulation of the production of the RANTES chemokine by endothelial cells. Synergistic induction by IFN-gamma plus TNF-alpha and inhibition by IL-4 and IL-13. J. Immunol. *154*, 1870-1878.

Marker, O. and Thomsen, A.R. (1986). T-cell effector function and unresponsiveness in the murine lymphocytic choriomeningitis virus infection. I. On the mechanism of a selective suppression of the virus-specific delayed-type hypersensitivity response. Scandinavian Journal of Immunology *24*, 127-135.

Martins, A.B. and Raffel, S. (1964) Cellular activities in hypersensitivity. I. Comparative cytology of delayed 'Jones-Mote' and Arthus reactions. J. Immunol. *93*, 937

Masinovsky, B., Urdal, D., and Gallatin, W.M. (1990). IL-4 acts synergistically with IL-1 beta to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1. J. Immunol. *145*, 2886-2895.

Mason, D. (1988). The roles of T cell subpopulations in allograft rejection. Transplant. Proc. *20*, 239-242.

Matzinger, P. (1994) Tolerance, danger, and the extended family. Ann. Rev. Immunol. *12*, 991-1045

McCallum, D.I. (1957) Histopathology of patch tests. Trans. St. John's Hosp. Dermatol. Soc. *39*, 11

McCarthy, S.A. and Singer, A. (1986). Recognition of MHC class I allodeterminants regulates the generation of MHC class II-specific CTL. J. Immunol. *137*, 3087-3092.

McKenzie, A.N.J., Culpepper, J.A., de Waal Malefyt, R., Brière, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B.G., Menon, S., de Vries, J.E., Banchereau, J., and Zurawski, G. (1993). Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. Proc. Natl. Acad. Sci. USA *90*, 3735-3739.

Meade, R., Askenase, P.W., Geba, G.P., Neddermann, K., Jacoby, R.O., and Pasternak, R.D. (1992). Transforming growth factor b1 inhibits murine immediate and Delayed Type Hypersensitivity. J. Immunol. *149*, 521-528.

Metaxas, M.N. and Metaxas-Buhler, M. (1955) Studies in the cellular transfer of tuberculin sensitivity in the guinea pig. J. Immunol. *75*, 333

Mihara, M., Ikuta, M., Koishihara, Y., and Ohsugi, Y. (1991). Interleukin 6 inhibits delayed type hypersensitivity and the development of adjuvant arthritis. Eur. J. Immunol. *21*, 2327-2331.

Miller, J.F., Vadas, M.A., Whitelaw, A., and Gamble, J. (1975). H-2 gene complex restricts transfer of delayed-type hypersensitivity in mice. Proc. Natl. Acad. Sci. USA *72*, 5095-5098.

Miller, J.F., Vadas, M.A., Whitelaw, A., and Gamble, J. (1976). Role of major histocompatibility complex gene products in delayed-type hypersensitivity. Proc. Natl. Acad. Sci. USA *73*, 2486-2490.

Miossec, P., Briolay, J., Dechanet, J., Wijdenes, J., Martinez-Valdez, H., and Banchereau, J. (1992). Inhibition of the production of proinflammatory cytokines and immunoglobulins by interleukin-4 in an ex vivo model of rheumatoid synovitis. Arthritis & Rheumatism *35*, 874-883.

Miyawaki, T., Suzuki, T., Butler, J.L., and Cooper, M.D. (1987). Interleukin-2 effects on human B cells activated in vivo. Journal of Clinical Immunology *7*, 277-287.

Mody, C.H., Paine, R., Jackson, C., Chen, G.H., and Toews, G.B. (1994). CD8 cells play a critical role in delayed type hypersensitivity to intact Cryptococcus neoformans. J. Immunol. *152*, 3970-3979.

Mond, J.J., Carman, J., Sarma, C., Ohara, J., and Finkelman, F.D. (1986). Interferon-gamma suppresses B cell stimulation factor (BSF-1) induction of class II MHC determinants on B cells. J. Immunol. *137*, 3534-3537.

Moore, K.W., O'Garra, A., de Waal Malefyt, R., Vieira, P., and Mosmann, T.R. (1993). Interleukin-10. Ann. Rev. Immunol. *11*, 165-190.

Moore, K.W., Vieira, P., Fiorentino, D.F., Hsu, D-H., de Waal Malefyt, R., Dang, M-N., Trounstine, M.L., Khan, T.A., Roncarolo, M-G., de Vries, J.E., Spits, H., and Mosmann, T.R. (1990a). Molecular biology of mouse and human cytokine synthesis inhibitory factor (CSIF; IL10). In Proceedings of the 1st International Congress on Cytokines: Basic Principles and Clinical Applications. S. Romagnani, ed. (Raven Press), pp. 137-144.

Moore, K.W., Vieira, P., Fiorentino, D.F., Trounstine, M.L., Khan, T.A., and Mosmann, T.R. (1990b). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science *248*, 1230-1234.

Morikawa, Y., Furotani, M., Kuribayashi, K., Matsuura, N., and Kakudo, K. (1992). The role of antigen-presenting cells in the regulation of delayed-type hypersensitivity. I. Spleen dendritic cells. Immunol. *77*, 81-87.

Morikawa, Y., Kuribayashi, K., and Saito, K. (1989). Immunoregulatory effect of antibody on delayed-type hypersensitivity in mice. International Archives of Allergy & Applied Immunology *90*, 130-136.

Morikawa, Y., Kuribayashi, K., and Saito, K. (1990). B-cell-mediated regulation of delayed-type hypersensitivity. Cell. Immunol. *131*, 338-351.

Morikawa, Y., Kuribayashi, K., Yoshikawa, F., Fujita, K., Mizushima, A., and Kakudo, K. (1991). The role of antibodies in the regulation of delayed-type hypersensitivity. Immunol. *74*, 146-152.

Mosmann, T.R. (1994a). Properties and functions of Interleukin 10. Adv. Immunol. *56*, 1-26.

Mosmann, T.R. (1994b). Interleukin 10. In The Cytokine Handbook. A.W. Thomson, ed. (London: Academic Press), pp. 223-237.

Mosmann, T.R. and Coffman, R.L. (1989). Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv. Immunol. *46*, 111-147.

Mosmann, T.R. and Sad, S. (1996). The expanding universe of T cell subsets - Th1, Th2 and more. Immunol. Today *17*, 138-146.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. *136*, 2348-2357.

Mosmann, T.R., Schumacher, J.H., Fiorentino, D.F., Leverah, J., Moore, K.W., and Bond, M.W. (1990). Isolation of MAbs specific for IL4, IL5, and IL6, and a new TH2-specific cytokine, Cytokine Synthesis Inhibitory Factor (CSIF, IL10), using a solid phase radioimmunoadsorbent assay. J. Immunol. *145*, 2938-2945.

Moulon, C., Vollmer, J. and Weltzien H.U. (1995). Characterization of processing requirements and metal cross-reactivities in T cell clones from patients with allergic contact dermatitis to mickel. Eur J Immunol. *25:* 3308-3315

Muhlradt, P.F. and Opitz, H.G. (1982). Clearance of interleukin 2 from the blood of normal and T cell-depleted mice. Eur. J. Immunol. *12*, 983-985.

Muller, I. (1992). Role of T cell subsets during the recall of immunologic memory to Leishmania major. Eur. J. Immunol. *22*, 3063-3069.

Muller, I., Pedrazzini, T., Kropf, P., Louis, J., and Milon, G. (1991). Establishment of resistance to Leishmania major infection in susceptible BALB/c mice requires parasite-specific CD8+ T cells. Int. Immunol. *3*, 587-597.

Muller, K.M., Jaunin, F., Masouye, I., Piguet, P.F., Saurat, J.H., and Hauser, C. (1995a). Involvement of granulocytes and the adhesion receptors intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 in tissue inflammation induced by Th2-type helper cells. J. Invest. Dermatol. *104*, 350-354.

Muller, K.M., Jaunin, F., Masouye, I., Saurat, J.H., and Hauser, C. (1993). Th2 cells mediate IL-4-dependent local tissue inflammation. J. Immunol. *150*, 5576-5584.

Muller, K.M., Lisby, S., Arrighi, J.F., Grau, G.E., Saurat, J.H., and Hauser, C. (1994). H-2D haplotype-linked expression and involvement of TNF-alpha in Th2 cell-mediated tissue inflammation. J. Immunol. *153*, 316-324.

Muller, K.M., Rocken, M., Carlberg, C., and Hauser, C. (1995b). The induction and functions of murine T-helper cell subsets. J. Invest. Dermatol. *105*, 8S-13S.

Mulligan, M.S., Jones, M.L., Vaporciyan, A.A., Howard, M.C., and Ward, P.A. (1993). Protective effects of IL-4 and IL-10 against immune complex-induced lung injury. J. Immunol. *151*, 5666-5674.

Muraguchi, A., Hirano, T., Tang, B., Matsuda, T., Horii, Y., Nakajima. K. and Kishimoto, T. (1988) The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. J. Exp. Med. *167*, 332-344.

Murphy, E.E., Terres, G., Macatonia, S.E., Hsieh, C.-S., Mattson, J., Lanier, L., Wysocka, M., Trinchieri, G., Murphy, K., and O'Garra, A. (1994). B7 and interleukin 12 cooperate for proliferation and interferon gamma production by mouse T helper clones that are unresponsive to B7 costimulation. J. Exp. Med. *180*, 223-231.

Nakamura, R.M., Goto, Y., Kitamura, K., and Tokunaga, T. (1989). Two types of suppressor T cells that inhibit delayed-type hypersensitivity to Mycobacterium intracellulare in mice. Infection & Immunity *57*, 779-784.

Nakamura, S., Sanui, H., and Nomoto, K. (1986). Relationship between the tuberculin-type and Jones-Mote-type hypersensitivities: suppression of basophil infiltration by mycobacterial adjuvant. Immunol. *58*, 397-403.

Nash, A.A. and Gell, P.G.H. (1981) The delayed hypersensitivity T cell and its interaction with other T cells. Immunol. Today *2*, 162-165.

Nathan, C.F., Murray, H.W., Wiebe, M.E., and Rubin, B.Y. (1983). Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. *158*, 670-689.

Nelson, D.S. (1964) The cutaneous reactivity of guinea pigs to pure protein antigens. II. The effects of different routes of injection of antigen and adjuvant. Int. Arch. Allergy *25*, 315

Nelson, D.S. and boyden, S.V. (1964) The cutaneous reactivity of guinea pigs to pure protein antigens. I. A critical evaluation of methods for the induction of delayed-type hypersensitivity to pure proteins. Int. Arch. Allergy *25*, 279

Ng, K.H., Watson, J.D., Prestidge, R., and Buddle, B.M. (1995). Cytokine mRNA expressed in tuberculin skin test biopsies from BCG-vaccinated and Mycobacterium bovis inoculated cattle. Immunology & Cell Biology *73*, 362-368.

Ohara, J. and Paul, W.E. (1985). Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. Nature *315*, 333-336.

Ohashi, P.S., Oehen, S., Aichele, P., Pircher, H., Odermatt, B., Herrera, P., Higuchi, Y., Buerki, K., Hengartner, H., and Zinkernagel, R.M. (1993). Induction of diabetes is influenced by the infectious virus and local expression of MHC class I and tumor necrosis factor-alpha. J. Immunol. *150*, 5185-5194.

Openshaw, P.J.M. (1995) Immunity and immunopathology to respiratory syncytial virus. Am. J. Respir. Crit. Care Med. *152*, s59

Ortaldo, J.R., Mason, A., and Overton, R. (1986). Lymphokine-activated killer cells. Analysis of progenitors and effectors. J. Exp. Med. *164*, 1193-1205.

Ortaldo, J.R., Mason, A.T., Gerard, J.P., Henderson, L.E., Farrar, W., Hopkins, R.F., Herberman, R.B., and Rabin, H. (1984). Effects of natural and recombinant IL 2 on regulation of IFN gamma production and natural killer activity: lack of involvement of the Tac antigen for these immunoregulatory effects. J. Immunol. *133*, 779-783.

Oswald, I.P., Gazzinelli, R.T., Sher, A., and James, S.L. (1992a). IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. J. Immunol. *148*, 3578-3582.

Oswald, I.P., Wynn, T.A., Sher, A., and James, S.L. (1992b). Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. Proc. Natl. Acad. Sci. USA *89*, 8676-8680.

Paganelli, R., Scala, E., Ansotegui, I.J., Ausiello, C.M., Halapi, E., Fanales-Belasio, E., D'Offizi, G., Mezzaroma, I., Pandolfi, F., Fiorilli, M., and et al, (1995). CD8+ T lymphocytes provide helper activity for IgE synthesis in human immunodeficiency virus-infected patients with hyper-IgE [see comments]. J. Exp. Med. *181*, 423-428.

Parish, C.R. (1972). The relationship between humoral and cell-mediated immunity. Transplant. Rev. *13*, 35-66.

Pastore, S., Cavani, A. and Girolomoni, G. (1996) Epidermal cytokine and neuronal peptide modulation of contact hypersensitivity reactions. Immunopharmacology *31*, 117

Pearce, E.J., Caspar, P., Grzych, J.M., Lewis, F.A., and Sher, A. (1991). Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni. J. Exp. Med. *173*, 159-166.

Philip, R. and Epstein, L.B. (1986). Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon and interleukin-1. Nature *323*, 86-89.

Picker, L.J., Singh, M.K., Zdraveski, Z., Treer, J.R., Waldrop, S.L., Bergstresser, P.R., and Maino, V.C. (1995). Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. Blood *86*, 1408-1419.

Pircher, H., Burki, K., Lang, R., Hengartner, H., and Zinkernagel, R.M. (1989). Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature *342*, 559-561.

Pirmez, C., Yamamura, M., Uyemura, K., Paes-Oliveira, M., Conceicao-Silva, F., and Modlin, R.L. (1993). Cytokine patterns in the pathogenesis of human leishmaniasis [see comments]. J. Clin. Invest. *91*, 1390-1395.

Pober, J.S. and Cotran, R.S. (1990a). The role of endothelial cells in inflammation. Transplantation *50*, 537-544.

Pober, J.S. and Cotran, R.S. (1990b). Cytokines and endothelial cell biology. Physiological Reviews *70*, 427-451.

Powrie, F., Menon, S., and Coffman, R.L. (1993). Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity *in vivo*. Eur. J. Immunol. *23*, 2223-2229.

Ptak, W., Bereta, M., Ptak, M., and Askenase, P.W. (1986). Isotype-like suppression of T cell-mediated immunity in vivo. I. Delayed-type hypersensitivity specificity of T cell suppression induced by antigen-binding T cell factors that initiate contact sensitivity. J. Immunol. *136*, 1554-1563.

Punnonen, J., Aversa, G., Cocks, B.G., McKenzie, A.N.J., Menon, S., Zurawski, G., de Waal Malefyt, R., and de Vries, J.E. (1993). Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc. Natl. Acad. Sci. USA *90*, 3730-3734.

Rabin, E.M., Mond, J.J., Ohara, J., and Paul, W.E. (1986). Interferon-gamma inhibits the action of B cell stimulatory factor (BSF)-1 on resting B cells. J. Immunol. *137*, 1573-1576.

Raffel S. and Newel, J.M. (1958) Delayed hypersensitivity induced by antigen-antibody complexes. J. Exp. Med. *108*, 823

Ranheim, E.A. and Kipps, T.J. (1993). Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J. Exp. Med. *177*, 925-935.

Rasmussen, R., Takatsu, K., Harada, N., Takahashi, T., and Bottomly, K. (1988). T cell-dependent hapten-specific and polyclonal B cell responses require release of interleukin 5. J. Immunol. *140*, 705-712.

Rennick, D., Yang, G., Muller-Sieburg, C., Smith, C., Arai, N., Takebe, Y., and Gemmell, L. (1987). Interleukin 4 (B-cell stimulatory factor 1) can enhance or antagonize the factor-dependent growth of hemopoietic progenitor cells. Proc. Natl. Acad. Sci. USA *84*, 6889-6893.

Richard, L., Forget, A., and Turcotte, R. (1990). Biological properties of factors secreted by antigen-reactive suppressor cells in mice infected with Mycobacterium lepraemurium. Infection & Immunity *58*, 3531-3536.

Richerson, H.B., Dvorak, H.F., and Leskowitz, S. (1970). Cutaneous basophil hypersensitivity. I. A new look at the Jones-Mote reaction, general characteristics. J. Exp. Med. *132*, 546-557.

Rivas, J.M. and Ullrich, S.E. (1992). Systemic suppression of delayed-type hypersensitivity by supernatants from UV-irradiated keratinocytes: An essential role for keratinocyte-derived IL-10. J. Immunol. *149*, 3865-3871.

Rivas, J.M. and Ullrich, S.E. (1994). The role of IL-4, IL-10, and TNF-alpha in the immune suppression induced by ultraviolet radiation. Journal of Leukocyte Biology *56*, 769-775.

Robinson, D.S., Hamid, Q., Jacobson, M., Ying, S., Kay, A.B., and Durham, S.R. (1993). Evidence for Th2-type T helper cell control of allergic disease in vivo. Springer Seminars in Immunopathology *15*, 17-27.

Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., Corrigan, C., Durham, S.R., and Kay, A.B. (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. New England Journal of Medicine *326*, 298-304.

Romagnani, S. (1991a). Human TH1 and TH2 subsets: doubt no more. Immunol. Today *12*, 256-257.

Romagnani, S. (1991b). Type 1 T helper and type 2 T helper cells: functions, regulation and role in protection and disease. Int. J. Clin. Lab. Res. *21*, 152-158.

Romagnani, S. (1994). Lymphokine production by human T cells in disease states. Ann. Rev. Immunol. *12*, 227-257.

Romani, L., Mencacci, A., Cenci, E., Spaccapelo, R., Mosci, P., Puccetti, P., and Bistoni, F. (1993). CD4+ subset expression in murine candidiasis. Th responses correlate directly with genetically determined susceptibility or vaccine-induced resistance. J. Immunol. *150*, 925-931.

Rosenberg, S.A. and Lotze, M.T. (1986). Cancer immunotherapy using interleukin-2 and interleukin-2-activated lymphocytes. Ann. Rev. Immunol. *4*, 681-709.

Rothman, P., Lutzker, S., Cook, W., Coffman, R., and Alt, F.W. (1988). Mitogen plus interleukin 4 induction of C epsilon transcripts in B lymphoid cells. J. Exp. Med. *168*, 2385-2389.

Rott, O., Fleischer, B., and Cash, E. (1994). Interleukin-10 prevents experimental allergic encephalomyelitis in rats. Eur. J. Immunol. *24*, 1434-1440.

Rouvier, E., Luciani, M.F., and Golstein, P. (1993). Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. J. Exp. Med. *177*, 195-200.

Sad, S. and Mosmann, T.R. (1994). Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. J. Immunol. *153*, 3514-3522.

Sad, S. and Mosmann, T.R. (1995). IL-4, in the absence of antigen stimulation, induces an anergy-like state in differentiated CD8$^+$ Tc1 cells: Loss of IL-2 synthesis and autonomous proliferation but retention of cytotoxicity and synthesis of other cytokines. J. Exp. Med. *182*, 1505-1515.

Sad, S., Krishnan, L., Bleackley, R.C., Kagi, D., Hengartner, H., and Mosmann, T.R. (1997). Cytotoxicity and weak CD40 ligand expression of CD8$^+$ type 2 cytotoxic T cells restrict their potential B cell helper activity. Eur. J. Immunol. *27*: 914-922

Sad, S., Marcotte, R., and Mosmann, T.R. (1995). Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ cells secreting TH1 or TH2 cytokines. Immunity 2, 271-279.

Sadick, M.D., Heinzel, F.P., Holaday, B.J., Pu, R.T., Dawkins, R.S., and Locksley, R.M. (1990). Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. J. Exp. Med. *171*, 115-127.

Salerno, A., Dieli, F., Sireci, G., Bellavia, A., and Asherson, L. (1995) Interleukin-4 is a critical cytokine in contact sensitivity. Immunology *84*, 404-409.

Salgame, P., Abrams, J.S., Clayberger, C., Goldstein, H., Convit, J., Modlin, R.L., and Bloom, B.R. (1991). Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science *254*, 279-282.

Sanderson, C.J. (1992). Interleukin-5, eosinophils, and disease. Blood *79*, 3101-3109.

Sano, Y. and Shimao, S. (1993). Evaluation of the antigen specificity of the cellular infiltrate at the delayed type hypersensitivity reaction site with the use of an antigen-specific T cell clone. Journal of Dermatological Science *5*, 25-31.

Sato, T., Sasahara, T., Nakamura, Y., Osaki, T., Hasegawa, T., Tadakuma, T., Arata, Y., Kumagai, Y., Katsuki, M. and Habu, S. (1994) Naive T cells can mediated delayed-type hypersensitivity response in T cell receptor transgenic mice. Eur. J. Immunol. *24*, 1512-1516.

Schall, T.J. (1994) The chemokines. In: The cytokine handbook, edited by Thomson, A.W. London: Academic Press, p,419-460

Schleimer, R.P., Sterbinsky, S.A., Kaiser, J., Bickel, C.A., Klunk, D.A., Tomioka, K., Newman, W., Luscinskas, F.W., Gimbrone, M.A.,Jr., McIntyre, B.W., and et al, (1992). IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. J. Immunol. *148*, 1086-1092.

Schreiber, S., Heinig, T., Thiele, H.G., and Raedler, A. (1995). Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease. Gastroenterology *108*, 1434-1444.

Schulz, M., Schuurman, H.J., Joergensen, J., Steiner, C., Meerloo, T., Kagi, D., Hengartner, H., Zinkernagel, R.M., Schreier, M.H., Burki, K., and et al, (1995). Acute rejection of vascular heart allografts by perforin-deficient mice. Eur. J. Immunol. *25*, 474-480.

Schwarz, A., Grabbe, S., Riemann, H., Aragane, Y., Simon, M., Manon, S., Andrade, S., Luger, T.A., Zlotnik, A., and Schwarz, T. (1994). In vivo effects of interleukin-10 on contact hypersensitivity and delayed-type hypersensitivity reactions. J. Invest. Dermatol. *103*, 211-216.

Scott, P. and Kaufmann, S.H. (1991). The role of T-cell subsets and cytokines in the regulation of infection. Immunol. Today *12*, 346-348.

Scott, P., Natovitz, P., Coffman, R.L., Pearce, E., and Sher, A. (1988). Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. J. Exp. Med. *168*, 1675-1684.

Scott, P., Pearce, E., Cheever, A.W., Coffman, R.L., and Sher, A. (1989). Role of cytokines and CD4+ T-cell subsets in the regulation of parasite immunity and disease. Immunol. Rev. *112*, 161-182.

Scovern, H. and Kantor, F.S. (1982). Local passive transfer of delayed-type hypersensitivity in the mouse. J. Immunol. *129*, 25-29.

Seder, R.A. and Paul, W.E. (1994). Acquisition of lymphokine-producing phenotype by CD4+ T cells. [Review]. Ann. Rev. Immunol. *12*, 635-673.

Seder, R.A., Boulay, J.L., Finkelman, F., Barbier, S., Ben Sasson, S.Z., Le Gros, G., and Paul, W.E. (1992). CD8+ T cells can be primed in vitro to produce IL-4. J. Immunol. *148*, 1652-1656.

Seder, R.A., Gazzinelli, R., Sher, A., and Paul, W.E. (1993). Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. Proc. Natl. Acad. Sci. USA *90*, 10188-10192.

Sher, A. and Coffman, R.L. (1992). Regulation of immunity to parasites by T cells and T cell-derived cytokines. Ann. Rev. Immunol. *10*, 385-409.

Sher, A., Gazzinelli, R.T., Oswald, I.P., Clerici, M., Kullberg, M., Pearce, E.J., Berzofsky, J.A., Mosmann, T.R., James, S.L., and Morse, H.C. (1992). Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. Immunol. Rev. *127*, 183-204.

Sher, A., Sacks, D.L., and Scott, P.A. (1983). Host and parasite factors influencing the expression of cutaneous leishmaniasis. Ciba Found. Symp. *99*, 174-189.

Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. (1992). Lymphocyte interactions with endothelial cells. Immunol. Today *13*, 106-112.

Shiohara, T., Nickoloff, B.J., Moriya, N., Gotoh, C., and Nagashima, M. (1988). In vivo effects of interferon-gamma and anti-interferon-gamma antibody on the experimentally induced lichenoid tissue reaction. British Journal of Dermatology *119*, 199-206.

Silber, A., Newman, W., Reimann, K.A., Hendricks, E., Walsh, D., and Ringler, D.J. (1994). Kinetic expression of endothelial adhesion molecules and relationship to leukocyte recruitment in two cutaneous models of inflammation. Laboratory Investigation *70*, 163-175.

Simon, J.C., Cruz, P.D., Bergstresser, P.R., and Tigelaar, R.E. (1990). Low Dose Ultraviolet B-Irradiated Langerhans Cells Preferentially Activate Cd4+ Cells of the T-Helper-2 Subset. J. Immunol. *145*, 2087-2091.

Simon, J.C., Mosmann, T., Edelbaum, D., Schopf, E., Bergstresser, P.R., and Cruz, P.D.,Jr. (1994). In vivo evidence that ultraviolet B-induced suppression of allergic contact sensitivity is associated with functional inactivation of Th1 cells. Photodermatology,Photoimmunology & Photomedicine *10*, 206-211.

Sironi, M., Munoz, C., Pollicino, T., Siboni, A., Sciacca, F.L., Bernasconi, S., Vecchi, A., Colotta, F., and Mantovani, A. (1993). Divergent effects of interleukin-10 on cytokine production by mononuclear phagocytes and endothelial cells. Eur. J. Immunol. *23*, 2692-2695.

Skoglund, C. and Scheynius, A. (1990). Effects of interferon-gamma treatment on the cutaneous DTH reaction in rats. Archives of Dermatological Research *282*, 318-324.

Skoglund, C., Scheynius, A., Holmdahl, R., and Van Der Meide, P.H. (1988). Enhancement of DTH reaction and inhibition of the expression of class II transplantation antigens by in vivo treatment with antibodies against gamma-interferon. Clinical & Experimental Immunology *71*, 428-432.

Smith, K.A. (1984). Interleukin 2. Ann. Rev. Immunol. *2*, 319-333.

Smith, S.R., Terminelli, C., Pennline, K.J., Kenworthy-Bott, L., Donkin, J., and Calzetta, A. (1995). Inhibitory effects of recombinant human interleukin 10 on disease manifestations in a P-->F1 model of acute graft versus host disease. Transplantation *59*, 890-896.

Snapper, C.M. and Paul, W.E. (1987a). B cell stimulatory factor-1 (interleukin 4) prepares resting murine B cells to secrete IgG1 upon subsequent stimulation with bacterial lipopolysaccharide. J. Immunol. *139*, 10-17.

Snapper, C.M. and Paul, W.E. (1987b). Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science *236*, 944-947.

Splawski, J.B., McAnally, L.M., and Lipsky, P.E. (1990). IL-2 dependence of the promotion of human B cell differentiation by IL-6 (BSF-2). J. Immunol. *144*, 562-569.

Springer, T.A. (1990). Adhesion receptors of the immune system. Nature *346*, 425-434.

Standiford, T.J., Strieter, R.M., Kasahara, K., and Kunkel, S.L. (1990). Disparate regulation of interleukin 8 gene expression from blood monocytes, endothelial cells, and fibroblasts by interleukin 4. Biochemical & Biophysical Research Communications *171*, 531-536.

Standiford, T.J., Strieter, R.M., Lukacs, N.W., and Kunkel, S.L. (1995). Neutralization of IL-10 increases lethality in endotoxemia. Cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor. J. Immunol. *155*, 2222-2229.

Stavitsky A.B. (1948) Passive cellular transfer of the tuberculin type of hypersensitivity. Proc. Soc. Exp. Biol. Med. *67*, 225

Stein, P., Dubois, P., Greenblatt, D., and Howard, M. (1986). Induction of antigen-specific proliferation in affinity-purified small B lymphocytes: requirement for BSF-1 by type 2 but not type 1 thymus-independent antigens. J. Immunol. *136*, 2080-2089.

Stern, M., Meagher, L., Savill, J., and Haslett, C. (1992). Apoptosis in human eosinophils. Programmed cell death in the eosinophil leads to phagocytosis by macrophages and is modulated by IL-5. J. Immunol. *148*, 3543-3549.

Stevenhagen, A. and van Furth, R. (1993). Interferon-gamma activates the oxidative killing of Candida albicans by human granulocytes. Clinical & Experimental Immunology *91*, 170-175.

Strack, P., Martin, C., Saito, S., DeKruyff, R.H., and Ju, S.T. (1990). Metabolic inhibitors distinguish cytolytic activity of CD4 and CD8 clones. Eur. J. Immunol. *20*, 179-184.

Strieter, R.M., Standiford, T.J., Huffnagle, G.B., Colletti, L.M., Lukacs, N.W., and Kunkel, S.L. (1996). "The good, the bad, and the ugly." The role of chemokines in models of human disease. J. Immunol. *156*, 3583-3586.

Stuart, P.M., Zlotnik, A., and Woodward, J.G. (1988). Induction of class I and class II MHC antigen expression on murine bone marrow-derived macrophages by IL-4 (B cell stimulatory factor 1). J. Immunol. *140*, 1542-1547.

Susskind, B., Shornick, M.D., Iannotti, M.R., Dufffy, B., Tanden, P.M.N., Siegel, J.P. and Mohanakumart, T. (1996) Cytolytic effector mechanisms of human CD4+ cytotoxic T lymphocytes. Human Immunol. *45*, 64

Swain, S.L., McKenzie, D.T., Dutton, R.W., Tonkonogy, S.L., and English, M. (1988). The role of IL4 and IL5: characterization of a distinct helper T cell subset that makes IL4 and IL5 (Th2) and requires priming before induction of lymphokine secretion. Immunol. Rev. *102*, 77-105.

Swain, S.L., Weinberg, A.D., and English, M. (1990a). CD4+ T cell subsets : Lymphokine secretion of memory cells and of effector cells which develop from precursors in vitro. J. Immunol. *144*, 1788-1799.

Swain, S.L., Weinberg, A.D., English, M., and Huston, G. (1990b). IL-4 Directs the Development of Th2-Like Helper Effectors. J. Immunol. *145*, 3796-3806.

Sy, M., Moorhead, J.W. and Claman, H.N. (1979) Regulation of cell mediated immunity by antibodies: possible role of anti-receptor antibodies in the regulation of contact sensitivity to DNFB in Mice. J. Immunol. *123*, 2593-2598.

Sztein, M.B., Steeg, P.S., Johnson, H.M., and Oppenheim, J.J. (1984). Regulation of human peripheral blood monocyte DR antigen expression in vitro by lymphokines and recombinant interferons. J. Clin. Invest. *73*, 556-565.

Taguchi, T., McGhee, J.R., Coffman, R.L., Beagley, K.W., Eldridge, J.H., Takatsu, K., and Kiyono, H. (1990). Analysis of Th1 and Th2 Cells in Murine Gut-Associated Tissues - Frequencies of Cd4+ and Cd8+ T-Cells That Secrete IFN-Gamma and Il-5. J. Immunol. *145*, 68-77.

Tamaki, K. and Iijima, M. (1989). The effect of ultraviolet B irradiation on delayed-type hypersensitivity, cytotoxic T lymphocyte activity, and skin graft rejection. Transplantation *47*, 372-376.

Taub, D.D. and Cox, G.W. (1995). Murine Th1 and Th2 cell clones differentially regulate macrophage nitric oxide production. Journal of Leukocyte Biology *58*, 80-89.

Te Velde, A.A., de Waal Malefijt, R., Huijbens, R.J., de Vries, J.E., and Figdor, C.G. (1992). IL-10 stimulates monocyte Fc gamma R surface expression and cytotoxic activity. Distinct regulation of antibody-dependent cellular cytotoxicity by IFN-gamma, IL-4, and IL-10. J. Immunol. *149*, 4048-4052.

Te Velde, A.A., Huijbens, R.J., de Vries, J.E., and Figdor, C.G. (1990). IL-4 decreases Fc gamma R membrane expression and Fc gamma R-mediated cytotoxic activity of human monocytes. J. Immunol. *144*, 3046-3051.

Tepper, R.I., Coffman, R.L., and Leder, P. (1992). An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. Science *257*, 548-551.

Tepper, R.I., Pattengale, P.K., and Leder, P. (1989). Murine interleukin-4 displays potent anti-tumor activity in vivo. Cell *57*, 503-512.

Thompson Snipes, L., Dhar, V., Bond, M.W., Mosmann, T.R., Moore, K.W., and Rennick, D.M. (1991). Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. J. Exp. Med. *173*, 507-510.

Thomsen, A.R. and Marker, O. (1986). T-cell effector function and unresponsiveness in the murine lymphocytic choriomeningitis virus infection. II. Delayed-type hypersensitivity unresponsiveness reflects a defective differentiation from TD precursor to effector cell. Scandinavian Journal of Immunology *24*, 137-145.

Thomson, J.A., Troutt, A.B., and Kelso, A. (1993). Contact sensitization to oxazolone: involvement of both interferon-gamma and interleukin-4 in oxazolone-specific Ig and T-cell responses. Immunol. *78*, 185-192.

Thornhill, M.H., Kyan-Aung, U., and Haskard, D.O. (1990). IL-4 increases human endothelial cell adhesiveness for T cells but not for neutrophils. J. Immunol. *144*, 3060-3065.

Thornhill, M.H., Wellicome, S.M., Mahiouz, D.L., Lanchbury, J.S., Kyan-Aung, U., and Haskard, D.O. (1991). Tumor necrosis factor combines with IL-4 or IFN-gamma to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and -independent binding mechanisms. J. Immunol. *146*, 592-598.

Titus, R.G., Muller, I., Kimsey, P., Cerny, A., Behin, R., Zinkernagel, R.M., and Louis, J.A. (1991). Exacerbation of experimental murine cutaneous leishmaniasis with CD4+ Leishmania major-specific T cell lines or clones which secrete interferon-gamma and mediate parasite-specific delayed-type hypersensitivity. Eur. J. Immunol. *21*, 559-567.

Torres, B.A., Farrar, W.L., and Johnson, H.M. (1982). Interleukin 2 regulates immune interferon (IFN gamma) production by normal and suppressor cell cultures. J. Immunol. *128*, 2217-2219.

Tough, D.F. and Sprent, J. (1994). Turnover of Naive- and memory-phenotype T cells. J. Exp. Med. *179*, 1127-1135.

Townsend, A.R., Rothbard, J., Gotch, F.M., Bahadur, G., Wraith, D., and McMichael, A.J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell *44*, 959-968.

Trial, J. (1989) Adoptive transfer of early and late delayed-type hypersensitivity reactions mediated by human T cells. Reg. Immunol. *2*, 14-21.

Trinchieri, G., Matsumoto Kobayashi, M., Clark, S.C., Seehra, J., London, L., and Perussia, B. (1984). Response of resting human peripheral blood natural killer cells to interleukin 2. J. Exp. Med. *160*, 1147-1169.

Tsicopoulos, A., Hamid, Q., Varney, V., Ying, S., Moqbel, R., Durham, S.R., and Kay, A.B. (1992). Preferential messenger RNA expression of Th1-type cells (IFN-gamma+, IL-2+) in classical delayed-type (tuberculin) hypersensitivity reactions in human skin. J. Immunol. *148*, 2058-2061.

Tsuji, K., Nakahata, T., Takagi, M., Kobayashi, T., Ishiguro, A., Kikuchi, T., Naganuma, K., Koike, K., Miyajima, A., Arai, K., and et al, (1990). Effects of interleukin-3 and interleukin-4 on the development of "connective tissue-type" mast cells: interleukin-3 supports their survival and interleukin-4 triggers and supports their proliferation synergistically with interleukin-3. Blood *75*, 421-427.

Turk, J.L. (1980). Delayed Hypersensitivity (Amsterdam: Elsevier).

Turk, J.L. and Oort, J. (1963) A histological study of the early stages of the development of the tuberculin reaction after passive transfer of cells labelled with 3H-thymidine. Immunology *6*, 140.

Turk, J.L. and Polak, L. (1967). Studies on the origin and reactive ability in vivo of peritoneal exudate cells in delayed hypersensitivity. International Archives of Allergy & Applied Immunology *31*, 403-416.

Turk, J.L., Heather, C.J. and Diengdoh, J.V. (1966a) A histochemical analysis of mononuclear cell infiltrates of the skin with particular reference to delayed hypersensitivity in the guinea pig. Int. Arch. Allergy *29*, 278

Turk, J.L., Rudner, E.J., and Heather, C.J. (1966b). A histochemical analysis of mononuclear cell infiltrates of the skin. II. Delayed hypersensitivity in the human. International Archives of Allergy & Applied Immunology *30*, 248-256.

Udaka, K., Takeuchi, Y., and Movat, H.Z. (1970). Simple method for quantitation of enhanced vascular permeability. Proc. Soc. Exp. Biol. Med. *133*, 1384-1387.

Urban, J.F., Katona, I.M., Paul, W.E. and Finkelman, F.D. (1991) Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. Proc. Natl., Acad., Sci. USA *88*, 5513-5517.

Urban, J.F.,Jr., Madden, K.B., Cheever, A.W., Trotta, P.P., Katona, I.M., and Finkelman, F.D. (1993). IFN inhibits inflammatory responses and protective immunity in mice infected with the nematode parasite, Nippostrongylus brasiliensis. J. Immunol. *151*, 7086-7094.

Valle, A., Aubry, J.P., Durand, I., and Banchereau, J. (1991). IL-4 and IL-2 upregulate the expression of antigen B7, the B cell counterstructure to T cell CD28: an amplification mechanism for T-B cell interactions. Int. Immunol. *3*, 229-235.

Valle, A., Zuber, C.E., Defrance, T., Djossou, O., De Rie, M., and Banchereau, J. (1989). Activation of human B lymphocytes through CD40 and interleukin 4. Eur. J. Immunol. *19*, 1463-1467.

Van Loveren, H., Meade, R., and Askenase, P.W. (1983). An early component of delayed-type hypersensitivity mediated by T cells and mast cells. J. Exp. Med. *157*, 1604-1617.

Van Reijsen, F.C., Bruijnzeel-Koomen, C.A., Kalthoff, F.S., Maggi, E., Romagnani, S., Westland, J.K., and Mudde, G.C. (1992). Skin-derived aeroallergen-specific T-cell clones of Th2 phenotype in patients with atopic dermatitis. J. Allergy. Clin. Immunol. *90*, 184-193.

van der Heijden, F.L., Wierenga, E.A., Bos, J.D., and Kapsenberg, M.L. (1991). High frequency of IL-4-producing CD4+ allergen-specific T lymphocytes in atopic dermatitis lesional skin. J. Invest. Dermatol. *97*, 389-394.

Vercelli, D., Jabara, H.H., Arai, K., Yokota, T. and Geha, R.S. (1989) Endogenous interleukin 6 plays an obligatory role in interleukin 4-dependent human IgE synthesis. Eur, J. Immunol. *19*, 1419-1424.

Vieira, P., de Waal Malefyt, R., Dang, M.N., Johnson, K.E., Kastelein, R., Fiorentino, D.F., DeVries, J.E., Roncarolo, M.G., Mosmann, T.R., and Moore, K.W. (1991). Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. Proc. Natl. Acad. Sci. USA *88*, 1172-1176.

Waksman, B.H. and Matolsky, M. (1958) Quantitative study of local passive transfer of tuberculin sensitivity with peritoneal exudate cells in the guinea pig. J. Immunol. *81*, 235

Walsh, C.M., Hayashi, F., Saffran, D.C., Ju, S.T., Berke, G., and Clark, W.R. (1996). Cell-mediated cytotoxicity results from, but may not be critical for, primary allograft rejection. J. Immunol. *156*, 1436-1441.

Walsh, C.M., Matloubian, M., Liu, C.C., Ueda, R., Kurahara, C.G., Christensen, J.L., Huang, M.T., Young, J.D., Ahmed, R., and Clark, W.R. (1994). Immune function in mice lacking the perforin gene. Proc. Natl. Acad. Sci. USA *91*, 10854-10858.

Walsh, G.M., Hartnell, A., Wardlaw, A.J., Kurihara, K., Sanderson, C.J., and Kay, A.B. (1990). IL-5 enhances the in vitro adhesion of human eosinophils, but not neutrophils, in a leucocyte integrin (CD11/18)-dependent manner. Immunol. *71*, 258-265.

Wang, J.M., Rambaldi, A., Biondi, A., Chen, Z.G., Sanderson, C.J., and Mantovani, A. (1989). Recombinant human interleukin 5 is a selective eosinophil chemoattractant. Eur. J. Immunol. *19*, 701-705.

Wang, P., Wu, P., Anthes, J.C., Siegel, M.I., Egan, R.W., and Billah, M.M. (1994). Interleukin-10 inhibits interleukin-8 production in human neutrophils. Blood *83*, 2678-2683.

Warren, M.K. and Vogel, S.N. (1985). Opposing effects of glucocorticoids on interferon-gamma-induced murine macrophage Fc receptor and Ia antigen expression. J. Immunol. *134*, 2462-2469.

Wegmann, T.G., Lin, H., Guilbert, L.J., and Mosmann, T.R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: Is successful pregnancy a TH2 phenomenon? Immunol. Today *14*, 353-356.

Weissmann, G. (1989). The role of neutrophils in vascular injury: a summary of signal transduction mechanisms in cell/cell interactions. Springer Seminars in Immunopathology *11*, 235-258.

Willems, F., Marchant, A., Delville, J.P., Gerard, C., Delvaux, A., Velu, T., de Boer, M., and Goldman, M. (1994). Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. Eur. J. Immunol. *24*, 1007-1009.

Williams, N.S. and Engelhard, V.H. (1996). Identification of a population of CD4+ CTL that utilizes a perforin- rather than a Fas ligand-dependent cytotoxic mechanism. J. Immunol. *156*, 153-159.

Wogensen, L., Huang, X., and Sarvetnick, N. (1993). Leukocyte extravasation into the pancreatic tissue in transgenic mice expressing interleukin 10 in the islets of Langerhans. J. Exp. Med. *178*, 175-185.

Wong, R.L., Ruddle, N.H., Padula, S.J., Lingenheld, E.G., Bergman, C.M., Rugen, R.V., Epstein, D.I., and Clark, R.B. (1988). Subtypes of helper cells. Non-inflammatory type 1 helper T cells [published erratum appears in J Immunol 1989 Feb 1;142(3):1060]. J. Immunol. *141*, 3329-3334.

Wu, J., Cunha, F.Q., Liew, F.Y., and Weiser, W.Y. (1993). IL-10 inhibits the synthesis of migration inhibitory factor and migration inhibitory factor-mediated macrophage activation. J. Immunol. *151*, 4325-4332.

Wynn, T.A., Eltoum, I., Cheever, A.W., Lewis, F.A., Gause, W.C., and Sher, A. (1993). Analysis of cytokine mRNA expression during primary granuloma formation induced by eggs of Schistosoma mansoni. J. Immunol. *151*, 1430-1440.

Xu, H., DiIulio, N.A., and Fairchild, R.L. (1996). T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8+ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4+ T cells. J. Exp. Med. *183*, 1001-1012.

Xu, H., Gonzalo, J.A., St Pierre, Y., Williams, I.R., Kupper, T.S., Cotran, R.S., Springer, T.A., and Gutierrez-Ramos, J.C. (1994). Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. J. Exp. Med. *180*, 95-109.

Xu, Y.H., Macedonia, J., Sher, A., Pearce, E., and Cheever, A.W. (1991). Dynamic analysis of splenic Th1 and Th2 lymphocyte functions in mice infected with Schistosoma japonicum. Infect. Immun. *59*, 2934-2940.

Yagi, H., Tokura, Y., Wakita, H., Furukawa, F., and Takigawa, M. (1996). TCRV beta 7+ Th2 cells mediate UVB-induced suppression of murine contact photosensitivity by releasing IL-10. J. Immunol. *156*, 1824-1831.

Yamaguchi, Y., Hayashi, Y., Sugama, Y., Miura, Y., Kasahara, T., Kitamura, S., Torisu, M., Mita, S., Tominaga, A., and Takatsu, K. (1988). Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor. J. Exp. Med. *167*, 1737-1742.

Yamamura, M., Uyemura, K., Deans, R.J., Weinberg, K., Rea, T.H., Bloom, B.R., and Modlin, R.L. (1991). Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science *254*, 277-279.

Yano, S., Sone, S., Nishioka, Y., Mukaida, N., Matsushima, K., and Ogura, T. (1995). Differential effects of anti-inflammatory cytokines (IL-4, IL-10 and IL-13) on tumoricidal and chemotactic properties of human monocytes induced by monocyte chemotactic and activating factor. Journal of Leukocyte Biology *57*, 303-309.

Yasukawa, M., Yakushijin, Y., and Fujita, S. (1996). Two distinct mechanisms of cytotoxicity mediated by herpes simplex virus-specific CD4+ human cytotoxic T cell clones. Clinical Immunology & Immunopathology *78*, 70-76.

Yokota, T., Coffman, R.L., Hagiwara, H., Rennick, D.M., Takebe, Y., Yokota, K., Gemmell, L., Shrader, B., Yang, G., Meyerson, P., Luh, J., Hoy, P., Pene, J., Briere, F., Spits, H., Banchereau, J., de Vries, J.E., Lee, F., Arai, N., and Arai, K. (1987). Isolation and characterization of lymphokine cDNA clones encoding mouse and human

IgA-enhancing factor and eosinophil colony-stimulating factor activities: Relationship to interleukin 5. Proc. Natl. Acad. Sci. USA *84*, 7388-7392.

Yssel, H., de Waal Malefyt, R., Roncarolo, M.G., Abrams, J.S., Lahesmaa, R., Spits, H., and de Vries, J.E. (1992). IL-10 is produced by subsets of human CD4+ T cell clones and peripheral blood T cells. J. Immunol. *149*, 2378-2384.

Zaloom, Y., Walsh, L.P., McCulloch, P., and Gallagher, G. (1991). Enhancement of a delayed hypersensitivity reaction to a contact allergen, by the systemic administration of interleukin-2. Immunol. 72, 584-587.

Zheng, H., Fletcher, D., Kozak, W., Jiang, M., Hofmann, K.J., Conn, C.A., Soszynski, D., Grabiec, C., Trumbauer, M.E., Shaw, A., and et al, (1995). Resistance to fever induction and impaired acute-phase response in interleukin-1 beta-deficient mice. Immunity *3*, 9-19.

Zheng, H., Foteder, A., Singh, B. and Green D.R. (1989) An antigen-specific immunoregulatory activity from a helper T cell hybridoma: Possible relationship to the T cell receptor. In : Cellular Basis of Immune Modulation, edited by Kaplan, J.G., Green, D.R. and Bleakley, R.C. New York: Alan R. Liss, Inc., p. 379-382

Zimmerman, G.A., Prescott, S.M., and McIntyre, T.M. (1992). Endothelial cell interactions with granulocytes: tethering and signaling molecules. Immunol. Today *13*, 93-100.

Zinsser, H. (1925) Bacterial allergies and tissue reactions. Proc. Soc. Exp. Biol. Med. 22, 35

Zinsser, H. and Mueller, J.H. (1925) On the nature of bacterial allergies. J. Exp. Med. *41*, 159

Zuany-Amorim, C., Haile, S., Leduc, D., Dumarey, C., Huerre, M., Vargaftig, B.B., and Pretolani, M. (1995). Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. J. Clin. Invest. *95*, 2644-2651.

Zurawski, S.M., Mosmann, T.R., Benedik, M. and Zurawski, G. (1986) Alterations in the amino-terminal third of mouse interleukin 2: effects on biological activity and immunoreactivity. J. Immunol. *137*, 3354-3360.